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14. ABSTRACT
The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain-immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies. These studies are incorporating sufficient overlap of scientific content area to inform each other in a bench-to-bedside-to-bench approach. Results to date from the preclinical (animal) studies suggest a strong neuroinflammatory component to the illness model and provide leads for treatment development approaches in the animal model before translation to the clinic. Clinical study recruitment has begun and has shown preliminary correlations between proinflammatory cytokine markers and behavioral and neuroimaging outcomes. Larger samples sizes will continue to make these inter-relationships more clear.

15. SUBJECT TERMS
Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, cognitive deficits, pesticides, DFP, sarin

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain-immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, cognitive deficits, pesticides, DFP, sarin, CORT, genetics

3. OVERALL PROJECT SUMMARY:

INTRODUCTION

Background. More than 24 years after the 1991 Gulf War, 25-32% of the nearly 700,000 U.S. troops who served in the war still suffer from the debilitating symptomatic illness known as Gulf War Illness (GWI) (RAC, 2008, 2014, IOM, 2010). A growing body of evidence indicates that GWI is associated with diverse central nervous system (CNS) and immune alterations, but the specific pathobiological processes driving GWI symptoms have not been clearly elucidated (Zhang et al., 1999; Sullivan et al, 2003; Heaton et al., 2007; Toomey et al., 2009; Whistler et al., 2009; Broderick et al., 2011; Chao et al., 2011; Sullivan et al., 2013). Animal studies indicate that a chronic CNS inflammatory state can develop in response to an insult—chemical injury, infection, or physical trauma (including mild traumatic brain injury)—that mobilizes CNS defense systems via activation of glia, the brain's primary immune response cells, and release of chemical messengers that precipitate a complex of "sickness behavior symptoms" identified by measures of impaired memory and learning, increased pain sensitivity, and persistent fatigue, a symptom complex similar to that of GWI (Rathbone et al., 2015; Banks & Lein, 2012; Watkins et al., 2007; 2009; Zhang et al., 2010). Recent studies have also demonstrated CNS inflammatory effects of GW-related exposures and additional immune and cellular processes that plausibly explain the mechanisms contributing to the full spectrum of GWI symptoms (O'Callaghan et al., 2015; Milligan et al., 2009; Rivest et al., 2009; Spradling et al., 2011).

Consortium Management and Expertise. This multidisciplinary collaboration brings together established GWI researchers, and leading experts in brain-immune processes associated with neurotoxicology and neuroinflammation, damage to white matter and axonal transport, immunology, and immunogenetics. This team has designed a body of interrelated studies linked together by a cohesive model of brain-immune interactions as the basis for GWI. The consortium is led by Dr. Kimberly Sullivan, at Boston University (BU), whose extensive background in GWI research includes contributions in identifying effects of Gulf War exposures on brain structure and function (Sullivan et al., 2003; Sullivan et al., 2013; Yee et al., 2015). BU serves as the Coordinating Center for the Gulf War Illness Consortium (GWIC) and provides the Administrative and Data Management Cores (figure 1). The consortium also includes a Preclinical Core, consisting of experts at five sites who are working collaboratively to characterize the persistent neurological and immune effects of GW exposures at the physiological, tissue, and cellular levels. This is done in parallel with human studies conducted by the Clinical Core at three recruitment sites (and two additional laboratory sites) to characterize the specific profile of brain, immune, and genetic measures that distinguish veterans with GWI from healthy controls. The GWIC Steering Committee and External Advisory Committee monitors research progress and findings, and advises on research modifications and follow-up.

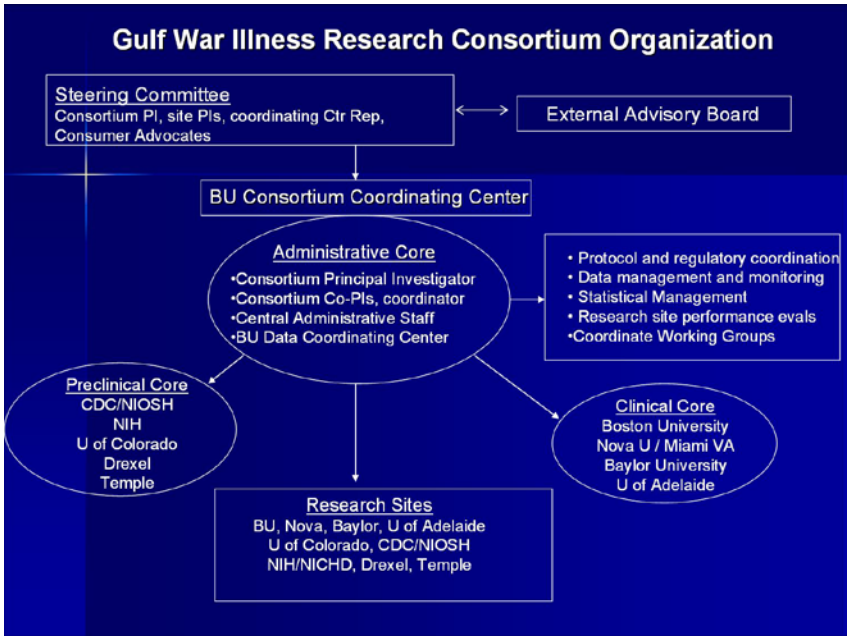


Figure 1. GWI Consortium schematic Organizational Chart

Objective. The primary objective of the Boston GWI consortium is to provide a cohesive understanding of the pathobiological mechanisms responsible for the symptoms of GWI in order to provide a rational and efficient basis for identifying beneficial treatments and diagnostic markers.

Research Plan. The consortium is undertaking a coordinated series of clinical and preclinical studies aimed at providing a comprehensive understanding of the pathobiology of GWI. This includes clinical case-control studies conducted in parallel at 3 subject recruitment sites—Boston, Miami, and Central Texas—that include a total of 300 Gulf War veterans. Clinical assessments include a) advanced neuroimaging protocols (MRI, DTI, fMRI, PET) that assess brain volumetrics, white matter integrity, and CNS inflammatory indicators, b) neuropsychological assessment of cognitive function, c) blood levels of cytokines and other immune signaling molecules, d) genetic expression of immune markers, e) pilot assessment of cerebrospinal fluid levels (CSF) of cytokines and neurotransmitters (in subgroup of Boston cohort), f) immunogenetic markers of innate immune responsivity, f) longitudinal assessment of brain-immune measures (Texas cohort only). Parallel preclinical studies are evaluating persistent effects of GW neurotoxicants *in vitro* and in rodent models of GWI. Preclinical studies are evaluating cellular effects of GW neurotoxicants on a) axonal transport, b) glial cytokine production, c) neurotransmitter signaling, d) myelination, and e) oligodendrocyte proliferation. Animal studies are determining the effects of GW exposures on: a) priming and maintaining glial activation, differentiating effects on astrocytes vs. microglia, b) glial activation in relation to development of learning impairment and chronic pain sensitivity, c) brain and blood levels of proinflammatory cytokines, and d) genetic expression of immune and inflammatory markers in brain and blood. Findings from clinical and

preclinical studies will be compared and used to identify specific brain-immune pathways that can be targeted for intervention by a variety of glial modulating and other currently available treatments. Treatment compounds will be tested in animal models to determine their effectiveness for resolving or ameliorating the pathobiological processes associated with GWI. Figure 2 represents the hypothesized mechanisms for GWI that will be tested by this planned series of preclinical and clinical experiments.

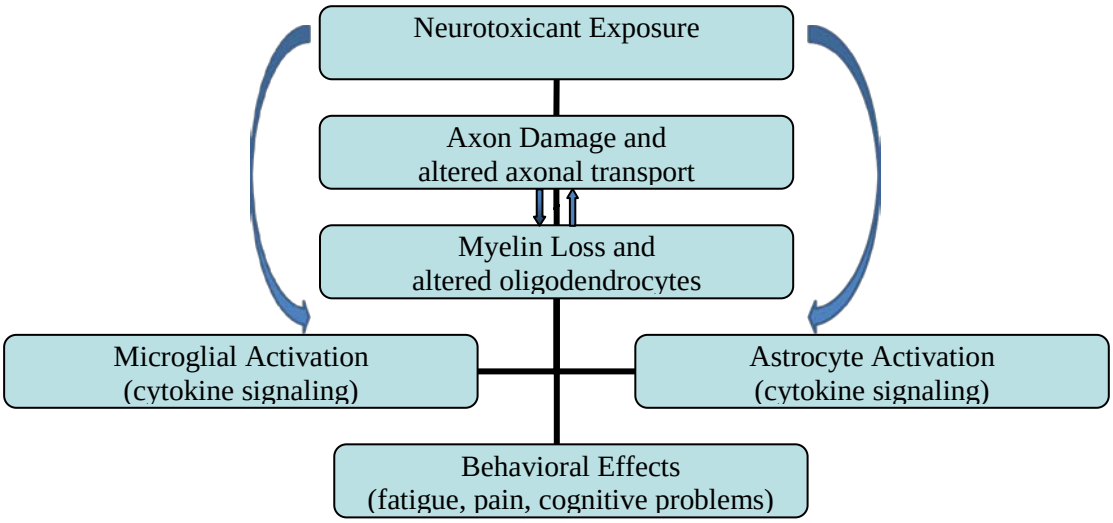
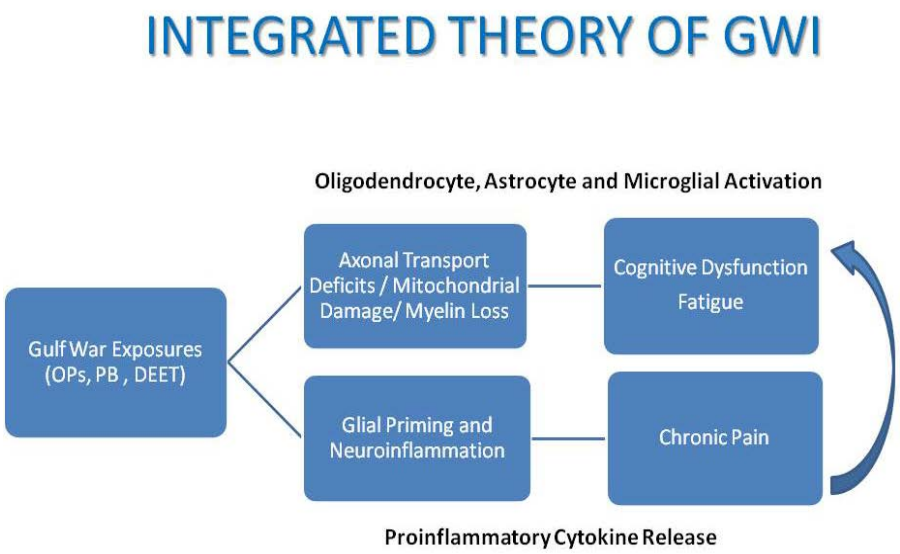


Figure 2. Schematic Representation of Hypothesized GWI Mechanisms

The GWI consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial *activation* and subsequent *priming* of glial responses that cause a chronic activation loop of stronger and longer proinflammatory effects between the immune system and the brain. Figure 3 below represents the integrated theory of GWI that will be tested in the consortium studies.



The overall aims of this integrated multidisciplinary consortium scientific focus are (1) To identify validated markers of GW illness by using state of the art neuroimaging, behavioral, genetic and blood markers of neuroinflammatory activation in both clinical and preclinical models that will elucidate targeted and validated treatment strategies (2) To create a Neuroinflammation Risk Profile for GWI (3) To identify viable mechanistic treatments based on identified pathophysiological pathways of GWI that have been validated in preclinical treatment models.

BODY

The approved statement of work for the entire study period is below:

STATEMENT OF WORK

Table 1. Brain-Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium

Task 1. Obtain necessary authorization prior to initiation of human subjects' and animal studies research (months 1-8)
1a. Attend pre-award meeting with CDMRP GWIRP program staff
1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements
1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals
1d. Obtain data use agreement from Hines VA for stored blood sample study
1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center for Disease Control/NIOSH, National Institutes of Health, Drexel University, Temple University and University of Colorado
1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members
Task 2. Preparation for consortium clinical studies (months 1-9)
2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.
2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.
2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies.
2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.
Task 3. Preparation for consortium preclinical studies (months 9 - 24)
3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-Colorado for planned studies of axonal transport, myelin integrity and learning and pain assessments.
3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.
Task 4. Perform preclinical cell and animal studies (months 9-42)
4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel - 30 Sprague Dawley rats, Temple - 27 Sprague Dawley rats).
4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (NIH – 624 NIH/S mice and 208 rats).
4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the

chronic nature of GWI (CDC – 100 C57BL/6 mice).
4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate "drugable" targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129-Cx3CR1 mice).
4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado – 120 rats).
4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from GWI neuroinflammatory rodent models (CDC – 60 rats, Nova).
4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado)(20 animals per treatment).
Task 5. Screening, recruitment and assessment of Gulf War veterans from three sites (months 9-42)
5a. Obtain informed consent from potentially eligible GW veterans
5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva samples.
5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis.
5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.
5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.
5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis
5g. Conduct preliminary analyses of clinical data
Task 6. Recruitment and assessment for Boston CSF and PET studies (months 24-42)
6a. Perform lumbar punctures to obtain cerebrospinal fluid markers of neuroinflammation in 50 GW veterans.
6b. Perform positron emission tomography (PET) scanning with novel EAAT2 ligand in partnership with RIO pharmaceuticals in 15 GW veterans.
6c. Perform FDG-PET scan imaging with 30 GW veterans after a computerized CPT cognitive challenge task.
Task. 7. Interim Analyses, Grant Submission, and Annual Reporting (Months 18-42)
7a. Data entry of all questionnaires, evaluations and quality control measures will be ongoing
7b. Interim Statistical analyses of data obtained from cognitive evaluations, blood markers, neuroimaging and questionnaire data will be performed periodically.
7c. Grant submissions to relevant funding agencies for further collaborative studies based on initial results and preliminary data targeted toward treatment strategies will be ongoing.
7d. Annual reports of progress will be written.
Task 8. Final analysis and Report Writing (months 42-48)
8a. Statistical analyses comparing brain MRI volumetrics, cognitive functioning, health symptom report and cytokine/chemokine markers in veterans with and without GWI

	8b. Statistical analyses of correlations between clinical and preclinical neuroinflammatory markers of GWI models
	8c. Perform longitudinal assessments of imaging, cognitive, health symptom and cytokine functioning in veterans with and without GWI
	8d. Perform validation analysis studies of identified biomarkers of GWI using an unrelated sample of stored blood and cognitive health symptom data from a prior CSP study.
	8e. Write final study report
	8f. Present findings at scientific meetings
	8g. Prepare manuscripts for submission
	8h. Write grant proposals based on consortium findings and identified treatment avenues for GWI.

The statement of work for year 2 is inclusive of Tasks 1-7 above. The statement of work for year 2 primarily describes the completion of the start-up phase of the 10 sub-studies including obtaining local and funder institutional review approvals for animal and clinical studies as well as establishing dosing models for cell and animal studies and finalizing clinical protocols for neuropsychological assessments, blood, saliva and CSF samples and neuroimaging sequences. In addition, in year 2, the plan was to have cell and animal studies underway and reporting initial results. The plan was also to begin subject recruitment for the clinical studies and to recruit 90 study participants for the study protocol including cognitive evaluations, interviews, neuroimaging and specimen collection. Progress toward completing each task is listed below.

TASK 1. OBTAIN NECESSARY AUTHORIZATION PRIOR TO INITIATION OF HUMAN SUBJECTS' AND ANIMAL STUDIES RESEARCH (MONTHS 1-8)

Task 1a. Attend pre-award meeting with CDMRP GWIRP program staff

Due to delays in funding the consortium as a result of the government shutdown, the pre-award meeting was held in February 2013 and was considered a post-award meeting. The meeting included an overview of study hypotheses and plans as well as a review of the consortium administrative and core center structure. The Consortium PI, Dr. Sullivan and other steering committee members were present at the meeting in addition to CDMRP commanders, grants officer's representative (GOR) and administrative staff. Required External Advisory Board (EAB) meetings have also begun to meet with the first meetings being held in September 2014, April 2015 and October 2015. The EAB provided helpful suggestions and comments for study progress and discussions for future meetings that will continue to occur bi-annually during the GWIC funding period.

Task 1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements

IRB documents have been submitted and approved for two of the clinical sites. Miami VA/NOVA University and Boston University (BU) have submitted IRB protocols and received local IRB approvals. Baylor University will submit initial IRB application shortly. University of Adelaide received exempt status from their local IRB.

Task 1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals

HRPO submissions have been submitted and approved for Miami VA/NOVA University and Boston University study protocols. The other clinical sites will be submitted as soon as local IRB approvals are obtained.

Task 1d. Obtain data use agreement from Hines VA for stored blood sample study
All relevant study forms from CSP 458 have been obtained and reviewed in order to generate a definition of CMI based on the Kansas definition. We are in the process of finalizing the definition after which we can compare how many subjects meet criteria for the CDC definition of CMI, the Kansas definition or both. This will inform our selection of the blood samples to analyze. Once selected, we can begin sending blood sample to Dr. Klimas for analysis at the Miami VA and submit the final draft of the DUA (we need to specify exactly what variables will be used in the DUA). We previously determined that the DUA needs to be in place prior to transfer of data from Hines VA Cooperative Studies Program Coordinating Center to BU Data Coordinating Center, but not prior to analyses on blood sample since those analyses will be done at a VA facility.

Task 1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center for Disease Control/NIOSH, National Institutes of Health, Drexel University, Temple University and University of Colorado
All local IACUC approvals have been obtained from CDC, NIH, Temple and University of Colorado. BU offsite IACUC approvals have been obtained for all animal study sites. ACURO final approvals have also been obtained for all pre-clinical sites and renewals are submitted for approvals as required.

Task 1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members
Hiring of local post-docs and Research assistants has been ongoing for each site. BUSPH has hired the consortium research assistant and Dr. O'Callaghan recently hired several post-doctoral associates. All current staff have completed IRB and IACUC trainings necessary for their work with animal and human studies.

TASK 2. PREPARATION FOR CONSORTIUM CLINICAL STUDIES
The consortium coordinating center and Administrative Core at Boston University has led many monthly web and in-person meetings to prepare for the clinical studies once all institutional approvals were obtained. A significant amount of time and effort was devoted to obtaining all required study and test administration materials and to developing centralized web-based data collection materials for the consortium studies. Table 2 lists these planning meetings. Smaller working group meetings were also held during the past year to plan for particular consortium topic areas. The Working Groups are described in Table 3. Since subject recruitment has begun, considerable time has been spent with training and quality control assurance meetings of clinical staff to ensure consistent inter-rater reliability and to reduce any administration drift from standard testing and scoring procedures.

Table 2. GWIC Monthly Planning and EAB Meetings

Date	Type of Meeting	Discussion Items
09-15-2014	In-person Ft. Detrick	➢ Presentations by GWIC PI and research investigators to DOD and External Advisory Board (EAB) and discussion
10-01-2014	Monthly Web-meeting	➢ Progress on Statement of Work
10-03-2014	In-person Boston	➢ Data management progress
11-05-2014	Monthly Web-meeting	➢ Pre-clinical and clinical study updates
12-11-2014	In-person Boston	➢ Data management progress
12-16-2014	Clinical and DCC	➢ Discussion of tracking system for specimen
12-17-2014	Monthly Web-Meeting	➢ Pre-clinical and clinical study updates
01-05-2015	In-person Boston	➢ Clinical group meeting with General Clinic Research Unit

01-07-2015	Monthly Web-meeting	➢ Progress on Statement of Work
02-11-2015	Monthly Web-meeting	➢ Pre-clinical and clinical study updates
03-04-2015	Monthly Web-meeting	➢ Pre-clinical and clinical study updates
03-12-2015	In-person Boston	➢ Data Coordinating Center updates
03-13-2015	Web-meeting, pre-clinical working group	➢ Pre-clinical working group meeting
04-01-2015	Web-meeting	➢ Pre-clinical and clinical study updates
04-08-2015	Web-based EAB meeting	➢ Presentations by GWIC PI and research investigators to DOD and External Advisory Board and discussion
05-06-2015	Web-meeting	➢ Recap of EAB meeting and suggestions ➢ Clinical and pre-clinical study updates
06-03-2015	Web-meeting	➢ Pre-clinical and clinical study updates
07-01-2015	Web-meeting	➢ Pre-clinical and clinical study updates
08-11-2015	In-person Boston	➢ Preparations for Boston meeting, pre-clinical and clinical study updates
08-12-2015	GWIC annual in person	➢ Presentations and updates from all research investigators
09-02-2015	Web-meeting	➢ Progress on Statement of Work
09-24-2015	In-person Boston	➢ Clinical group meeting to discuss lumbar puncture sub-study
10-07-2015	Web-meeting	➢ Discuss preparations for EAB meeting, clinical studies updates
10-28-2015	In-person Ft. Detrick	➢ Presentations by GWIC PI and research investigators to DOD and External Advisory Board

Table 3. Consortium Working Groups

Working Group	Tasks	Members
Data Management Service Group	Assist with QC issues, data cleaning and data management and sharing, website management.	Christine Chaisson, DCC Consortium PI, co-PIs
Statistics Service Group	Perform analyses and provides statistical planning and advice for study investigators and research site PIs.	Timothy Heeren, Christine Chaisson, Consortium PI/co-PIs
Translational Working Group	Forum for Intellectual property and material (IP) issues, translation of results into papers, abstracts, new grant submissions and how clinical and preclinical results can inform each other.	Michael Pratt – BU Tech Transfer office Consortium PI, co-PIs Research site PIs, RIO
Behavioral Studies Working Group	Plan imaging protocols and provide quality control for multiple imaging sites. Plan behavioral testing protocols and coordinate preclinical and clinical studies for comparability.	Drs. Sullivan, Killiany, Krengel, Toomey, Steele, Klimas, Coller, Hutchinson, Maier, Watkins
Histopathology Working Group	Plan tissue studies of proinflammatory, glial, axonal transport and mitochondrial markers in similarly dosed animal and cell models.	Drs. Baas, Black, O'Callaghan, Fields, Maier, Watkins
Immune Genetics Working Group	Plan and implement studies assessing brain-immune interactions involving glia and proinflammatory cytokines/chemokines through genetic SNPs and mRNA and miRNA protein studies.	Drs. Coller, Hutchinson, Klimas, Steele, Sullivan, Watkins, Maier

Task 2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.

Consortium website (http://sites.bu.edu/gwic/) is finalized and approval from each institution to use their logos was obtained. Electronic data collection forms using REDCap software and CATI recruitment software are now finalized and in use for subject screening and data collection. The study is utilizing Frontier Science's LDMS specimen tracking system for shipping samples to collaborating sites and for biorepository tracking. Training for the specimen tracking system has also been completed by all necessary staff. A refresher REDCap training was completed for all clinical staff and is in use as subjects are recruited and complete online questionnaires and screened for eligibility through CATI system.

Task 2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.

The BU Coordinating Center members has met several times during the year to slightly revise and discuss the cognitive administration and scoring manuals, specimen collection protocols and recruitment effort plans. These plans are finalized and the Behavioral Studies Working Group set up two in-person training days last August to train all clinical staff and to ensure proper quality control measures are in place for the clinical studies. This has been followed up by videotaping practice testing to ensure tester drift is not occurring and regular web meetings are being conducted with testing staff as subject recruitment gets underway to answer any questions or discuss problems with test administration/scoring issues. This has proven helpful to ensure consistent test administration and scoring at all study sites.

Table 4. Summary of Clinical Assessments and Tests Conducted at 3 Clinical Study Sites

	Boston		Miami		Texas		TOTAL
Data Collected	Cases	Controls	Cases	Controls	Cases	Controls	
Questionnaires (demographics, general health and symptoms,pain, fatigue, sleep, medical conditions, deployment/exposure history)	125	50	25	25	50	25	n = 300 (200 cases/100 controls)
Clinical evaluation (medical history, height, weight, supine/standing BP and pulse, psychiatric diagnostic assessment)	125	50	25	25	50	25	n = 300 (200 cases/100 controls)
Clinical lab tests (CBC, metabolic profile, lipid panel, TSH, ANA, RF)	125	50	25	25	50	25	n = 300 (200 cases/100 controls)
Research assays (plasma cytokines/chemokines, nanostring inflammasome genetic panel, and salivary cortisol)	125	50	25	25	50	25	n = 300 (200 cases/100 controls)
Neuroimaging (MRI volumetrics and relaxometry, DTI, fMRI)	125	50	-	-	50	25	n = 250 (175 cases/75 controls)
Neuropsychological assessment (executive function, attention, memory, psychomotor function, motivation, mood)	125	50	25*	25*	50	25	n= 300 (175 cases/75 controls)
Longitudinal assessment (clinical evaluation, clinical lab tests, plasma cytokines, neuroimaging, neuropsychological assessment)	-	-	-	-	50	25	n = 75 (50 cases/25 controls)

CBC=complete blood count, TSH=thyroid stimulating hormone, ANA=antinuclear antibodies, RF=rheumatoid factor, MRI=magnetic resonance imaging, DTI=diffusion tensor imaging * select neuropsychological tests will be done in Miami

Task 2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies

Training for researchers and clinical staff was completed at in-person meeting in Boston in August 2014 and continued to be monitored as described above. Working groups have finalized training procedures and protocols for cognitive, neuroimaging and laboratory procedures that are currently being used.

Task 2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.
The data use agreement was sent to VA and the requested edits have been made and the DUA sent back to VA for review. As soon as the DUA is approved, the blood samples will be sent to Dr. Klimas' lab at the Miami VA for analysis.

TASK 3. PREPARATION FOR CONSORTIUM PRECLINICAL STUDIES (MONTHS 9 - 24)
As previously described, monthly web meeting and working group meetings were ongoing during the past year to prepare for the planned preclinical studies and to coordinate overlap of the studies and to ensure that the same neurotoxicant dosing and exposure model of GWI was being used among the four preclinical study sites. The CDC site was tasked with comparing the mouse and rat models of GWI to ensure comparability for planned studies and to distribute dosed animals and animal tissue to the preclinical sites. The planning stage was successful as discussions progressed and initial studies began at the preclinical study sites. Preparation for the specific preclinical study sites are detailed below.

Task 3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-Colorado for planned studies of axonal transport, myelin integrity and learning and pain assessments.

Dr. O'Callaghan at the CDC site prepared and validated rat dosing models based on his initial mouse GWI dosing models from prior DOD funded studies using chronic daily corticosterone (CORT) and 1 dosage of the sarin-surrogate DFP (O'Callaghan et al., 2015). An initial set of rats were dosed and sent to the University of Colorado to initiate behavioral studies with the rat GWI dosing model (once a memorandum of understanding was approved between CDC and University of Colorado to ship the animals). The 8-day dosing model of CORT and DFP is listed below. Initial study results are also listed in the sub-studies below.

Both CDC and University of Colorado are attempting to compare behavioral findings in rats dosed at the University of Colorado and those rats dosed at CDC and shipped to Colorado. If results are comparable, investigators will use the most cost and time effective method, and the method that introduces the least amount of variables into the research to investigate the behavioral effects of DFP on rats. ACUC amendment for dosing DFP has recently been approved at University of Colorado for pain studies. The CDC is working with their own ACUC to allow for continued shipping of DFP-dosed rats and are gathering data to provide to Colorado vet and ACUC about the effects of dosing with DFP. CDC is working with their own veterinarian to answer questions about palliative care models that may be given to rats dosed with DFP to enhance survival before shipping.

Brain tissue from dosed rat and mouse GWI models were also sent to Drexel University and to NIH investigators to initiate the histopathology studies of potential neuronal, myelin, axonal and glial pathology and to Boston University investigators for brain MRI imaging analyses. Specifics of the dosing and brain extractions are listed below.

Sixty rats were perfused and their brains extracted and preserved in 10% formalin. Brains from rats in the control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5) were sent to Drexel University investigators. Three different cohorts of animals were

exposed to their respective group conditions and sets of sacrifices were performed at 12, 24, and 72 hours post DFP exposure. Animals in CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP).

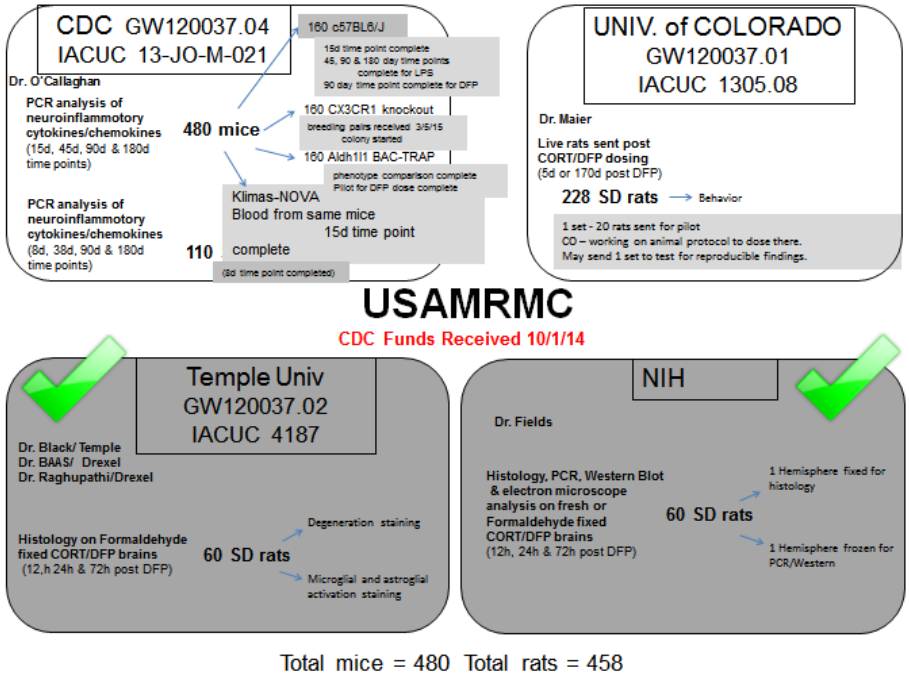


Figure 3. Distribution schematic of animals from CDC

The brains of fifty-five rats were removed and one hemisphere was flash frozen in dry ice and the other hemisphere was preserved in 10% formalin. CDC investigators sent brains from rats in the control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5) to Dr. Fields lab at NIH. Three different cohorts of animals were exposed to their respective group conditions and separate sacrifices were performed at 12, 24, and 72 hours post DFP exposure. Animals in CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP).

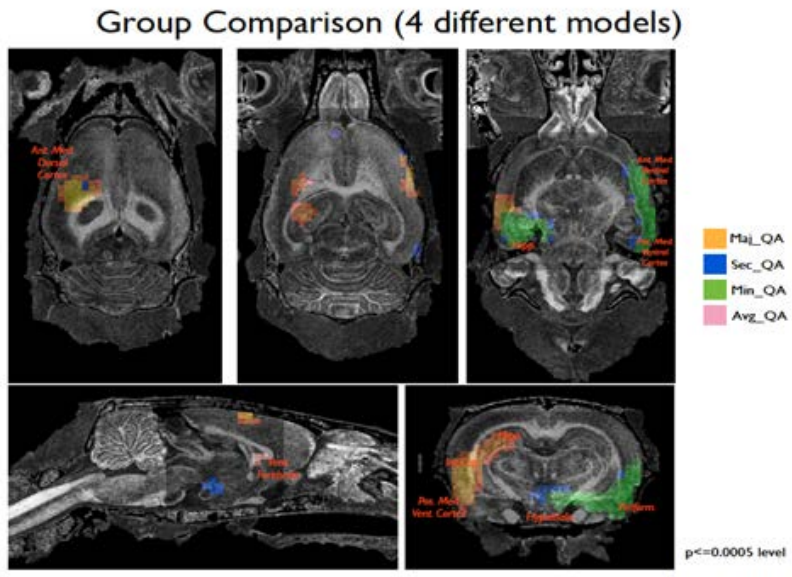
Current progress:

- On July 21st, CDC sacrificed 20 adult male rats [control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5)]. Animals in CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). Rats were perfused and brains were preserved in 10% formalin brains. Perfused brains were then sent out to Dr. Killiany and Boston University collaborators for brains to be structurally imaged.

- Following rat DFP exposure, CDC recorded specific behavioral outcomes of rats dosed with DFP in order to share details of the behavioral symptoms that follow DFP exposure with Colorado collaborators. Groups hope Colorado veterinary staff will be more amenable to dosing with DFP in

facilities in Colorado when they have more knowledge of SLUD symptoms in rodents following DFP exposure.

On July 23rd CDC shipped Drs. Sullivan and Killiany 20 perfused and extracted rat brain stored in 10% formalin. These brains were used for MRI diffusion tensor imaging (DTI) imaging from the group at Boston University. Brains from the 20 rats sent to BU included: control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5). Rats that were exposed to CORT were treated with 200mg/L CORT water for 4 days prior to dosing with saline or 1.5mg/kg DFP. Rats in the other 2 groups (control and DFP) were exposed to tap water for 4 days and then dosed with saline or DFP (1.5mg/kg), respectively. All rats were sacrificed 6 hours post-dosing and brains were shipped out the following day. The rat brains have been imaged at Boston University and imaging personnel were blind to the exposure status of the samples. Final post-processing and post-hoc analyses are pending however initial results suggest differences among the 4 groups. Further studies with later timepoints will aid in determining the significance of these early results.



The CDC original mouse model of GWI illness (O'Callaghan et al., 2015) displayed increased expression of pro-inflammatory cytokines following exposure to DFP and this effect was augmented by CORT pretreatment. This effect is seen at 6 hours post-dosing DFP.

Since rodent dosing of DFP in July, the CDC has not been permitted to dose any additional animals with DFP per request of the CDC veterinarian. The veterinarian has asked the O'Callaghan Lab to run a number of palliative care studies to see if DFP dosed animals can survive with easier access food or water and warming materials. CDC is not permitted to continue dosing with DFP until the veterinarian determines which type of palliative care will help animals survive exposure to DFP alone and ultimately determine humane end points. Once issues with vet and ACUC are resolved, CDC will dose rats in Morgantown to ship to the University of Colorado to compare behavioral findings in rats dosed at the University of Colorado and those rats dosed at CDC and shipped to Colorado. If results are comparable, investigators will use the most cost and time effective method, and the method that introduces the least amount of variables into the research to investigate the behavioral effects of DFP on rats.

Additionally, once DFP dosing issues are resolved, CDC will dose rats with DFP and CORT DFP and perfuse rats 7 days following DFP exposure. Brains of rats sacrificed at 7 days post-DFP exposure will be preserved in 10% formalin and will be sent to NIH investigators to initiate the planned histopathology and electron microscopy studies of potential neuronal, myelin, and axonal pathology. Rats will be placed in one of the 4 groups [control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5)]. Animals in CORT groups will be exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups will be given standard tap water for 4 days followed by a

single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). The 7 day time point will allow for investigation of neuronal changes or degradation at further time points post exposure.

Task 3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.

Dr. Dipankar Dutta was hired as a postdoctoral associate in the NIH laboratory of Dr. Douglas Fields. Dr. Dutta has been trained in making the cell cultures from embryonic mice and rats for studies of myelination and the co-cultures are currently being developed in the cell culture chambers for the ongoing and planned oligodendrocyte and myelin studies.

1. An assay to quantify the effects of GW toxicants on the process of myelination in-vitro is being developed. Cholinergic neurons are being myelinated in co-culture with oligodendrocytes, providing a framework to study the effects of GW agents on axonal myelination by oligodendrocytes. Techniques are being developed to produce purified motor neuron cultures. OPCs will now be combined with these neuronal cultures in multicompartment chambers for electrical stimulation of axons and treatment with pharmacological agents.
2. GW agents primarily disrupt cholinergic neurotransmission. Like neurons, oligodendrocytes express several muscarinic receptors, types 1-5, and preliminary research shows that oligodendroglia can respond to cholinergic stimulation. Dr. Field's lab has acquired and housed muscarinic receptor 1-5 KO mice, from which oligodendrocytes can be isolated and cultured with GW agents, so as to definitively delineate the role of these agents on oligodendrocyte biology and development.

NIH investigators have also received brain tissue from CDC to begin histopathology studies of myelin from exposed animals. The brains from rats exposed to neurotoxicants by Dr. O'Callaghan's lab were analyzed for changes in myelin and other proteins by western blot. This included 60 samples (brains), 12 treatment conditions, 3 time points, (12, 24 and 72 hr) for the proteins olig 2, MBP, and GAP-43. The results show that myelin proteins are affected, but the result is surprising. Rather than decreasing, as would be expected with myelin damage, we find an increase in myelin basic protein (MBP) levels increase significantly 72 hrs after treatment. This result, however, shows that myelinating glia are being affected by the treatments modeling exposures that patients with GWI are likely to have experienced. This is a very important finding with respect to the research on white matter damage found in GWI. We suspect that the increase in MBP at this time point may reflect an adaptive response to the toxicants in an attempt to recover from white matter injury. Histological analysis of the same brains will allow us to investigate this. A staff member was hired to do the microtome sectioning of these brains and immunocytochemical staining. This work is well underway and histological and immunocytochemical analysis will be shown in future updates.

Dr. Field's lab also investigated levels of olig2, a transcription factor for oligodendrocytes, to determine if oligodendrocytes are dying or increasing in number after treatment. No change in this protein was observed. Dr. Field's lab also tested for changes in GAP-43, because this protein is a marker for axon sprouting. One possibility for an increase in MBP after treatment might have been that axons were damaged and had begun to sprout in recovery. These sprouts would then need to be myelinated, which could account for the increase in MBP protein we found after treatment. This hypothesis was not supported. GAP-43 levels are not different among treatment groups.

Interestingly, the only treatment condition where MBP levels changed was in the CORT+DFP group. Neither DFP nor CORT alone caused a change. This result is consistent with other results that Dr. O'Callaghan has reported at CDC (O'Callaghan et al., 2015).

The next step will be to extend the treatment conditions beyond 72 hours and analyze MBP levels, because myelination is a long-term process and the shorter time points may not have captured the full response of what is occurring. These results will be reported in subsequent updates.

TASK 4. PERFORM PRECLINICAL CELL AND ANIMAL STUDIES (MONTHS 9-42)

The preclinical studies started later than initially anticipated for the CDC site and those sites waiting for distribution of animals or tissue from the CDC site because an interagency agreement for the release of funds from DOD to CDC took approximately 8 months out of the first year of funding to be released. Considering this delay in funding, a significant amount of work has been initiated at the 5 preclinical study sites to date. Specific progress to date is listed below for each of the sub-studies.

Task 4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel - 30 Sprague Dawley rats, Temple - 27 Sprague Dawley rats).

Studies to assess axonal transport and microtubule integrity *in vitro* and *in vivo* have been progressing as expected. Our research team continues to explore the effects of GW neurotoxins and cytokines on axonal transport and neuronal function. Items completed to date for the axonal transport and microtubule integrity group include:

1) An enhanced mitochondrial transport assay using a CMXRos mitochondrial dye which is sensitive to mitochondrial membrane potential, thus enabling us to not only appreciate mitochondrial transport but also the oxidative stress of the neuron. Preliminary experiments using this new dye are underway. Results from our previous mitotracker experiments suggest a bidirectional inhibition of mitochondrial transport after treatment with DFP.
2) CAMSAP2 experiments are complete and the protocol for CAMSAP2 is currently included in a paper under review at the Journal of Cell Biology. This technique is now being used as an additional outcome measure of how microtubule integrity is affected by Gulf War neurotoxins, as CAMSAP2 stabilizes the minus-ends of microtubules.
4) CORT and DFP working concentrations have been established and all assays have been optimized.
5) Studies using CORT + DFP experiments are underway. In these experiments, mature cortical and hippocampal neurons are pretreated with CORT prior to application of DFP at five concentrations (0.1 nM, 1 nM ,10 nM, 100 nM, and 1 μM). Live-cell imaging of mitochondrial transport, microtubule polymerization, and microtubule transport will be performed. A parallel set of experiments will be fixed and stained for acetylated tubulin and total tubulin. We will analyze the ratio of acetylated (stable microtubules) : total microtubule mass contained in neurons *in vitro* in control and treatment groups to better understand the loss of microtubule mass observed in previous experiments.
6) Our next step is to isolate and culture rat cortical astrocytes. Once cultures are established, astrocytes will be primed with CORT and treated with DFP. The culture supernatant will be collected and applied to purified cortical and hippocampal neurons to explore how cytokines secreted by astrocytes affect neurons *in vitro.* Parallel experiments will assess the cytokine profile under control and treatment conditions.
7) Histological studies on the *in vivo* rodent model reveal no obvious changes in total tubulin or acetylated tubulin in the brain compared to controls at an <u>acute</u> time point. This was expected as we previously observed no obvious signs of cell death or impaired axonal transport, which is consistent with toxin dosage that accurately reflects that to which the soldiers were exposed. Experiments will investigate the aforementioned parameters at chronic time points. In these experiments, immunostaining for microtubules (total and acetylated tubulin), axonal transport defects, and cell death will be explored.

8) Dr. Liang (Oscar) Qiang has arrived to Drexel University where he will use neurons and glia differentiated from GW veteran-derived induced pluripotent stem cells (see Figure 4 below).

Preliminary data from human-induced iPSC cells are shown below in Figure 4.

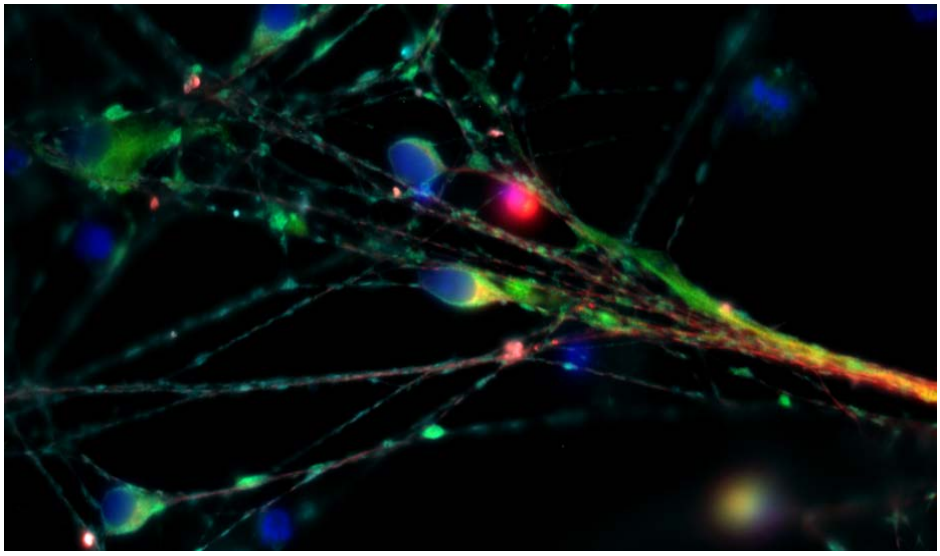


Figure 4. Image showing human IPS-derived neurons *in vitro.* Cells have been quadruple labeled with beta-3-tubulin (cyan), acetylated-tubulin (red), tyrosinated-tubulin (green), and DAPI (blue). Experiments documenting the effect of DFP treatment alone and after pre-treatment with cortisol are underway. Western blot experiments will be run in parallel.

Results to date indicate that CORT works in a similar fashion in in Vitro studies in Dr. Baas' lab as it does with animals in Dr. O'Callaghan's lab (see below).

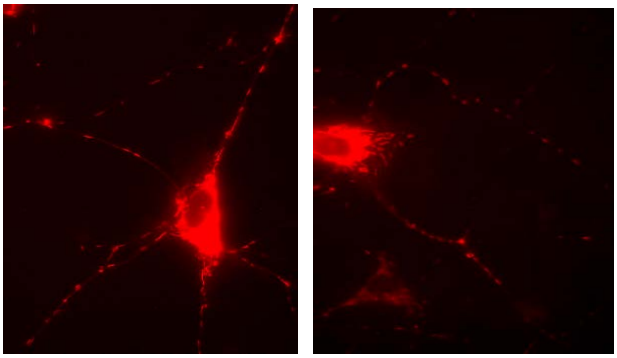


Figure 5. Images showing hippocampal neurons after treatment with DFP alone (left) or DFP after CORT pretreatment (right). The smaller mitochondria observed when DFP is applied after CORT pretreatment suggests a higher occurrence of mitochondrial fission. Transport is hindered in both cases.

The funded iPSC work is well connected to the consortium objectives as it is designed to assess axonal transport and microtubule dysfunction in GW relevant exposures including DFP and cortisol (human equivalent of CORT). The hypothesis is that exposure of neurons and/or neuroinflammatory cells to GW toxins caused long-lasting axonal transport/microtubule defects in neurons, and that these defects lead to a loss of microtubule mass, a change in the proportions of stable and labile microtubule mass, and/or flaws in the lattice of the microtubule that lead to abnormalities in how molecular motor proteins and other microtubule-related proteins interact with the microtubule and move down the axon. See results of animal cell studies that will be performed in human iPSC cells in Figure 3 below.

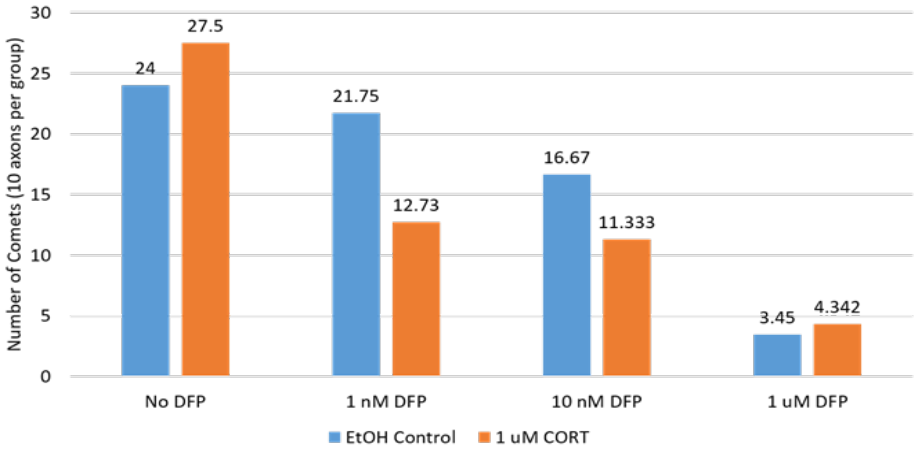


Figure 6. EB3 comet quantification under control or CORT-pretreatment conditions. CORT pretreatment has a profound effect on the average number of EB3 comets observed (i.e. the number of polymerizing microtubules) compared to DFP alone. These experiments were performed in rat hippocampal neurons and will be replicated in human-induced neurons in the funded iPSC study (GW140086).

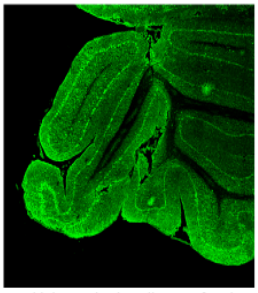
Task 4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (NIH – 624 NIH/S mice and 208 rats).

It has been determined that there are functional cholinergic receptors on oligodendrocytes and the site-PI Dr. Fields is characterizing the type of receptor and how they change during development, using immunocytochemistry and live cell calcium imaging.

1. We are characterizing the expression of muscarinic receptor type 1 through 5 in oligodendrocyte lineage cells in-vitro, in primary oligodendrocyte cultures and in co-culture with neurons. We are also characterizing their expression in the adult mice brain in-vivo. We observe that in oligodendrocytes, these receptors are differentially expressed in a complex manner, depending on differentiation stage of the cells, their location in the brain. Preliminary data also show that there are subcellular differences in receptor subtypes on oligodendroglial cells; for example on the cell processes vs. cell body (see figure). These initial data indicate that not all oligodendrocytes express these receptors in-vivo and the cells that do are closely associated with cholinergic projection systems in the brain.

Preliminary Results:

Cholinergic receptor expression on oligodendroglia *in vivo*



Using spinning disc confocal

II. Expression of muscarinic receptors 1-5 in various stages of oligodendrocyte development in-vitro.

Stages of the oligodendrocyte lineage*	Cellular compartments	Muscarinic Receptors				
		mAchR1	mAchR2	mAchR3	mAchR4	mAchR5
Bipolar OPCs (PDGFRa+)	Cell body	+++	+++	++	+	+++
	Cell processes	+++	+	++	-	++
Multipolar OPCs (NG2+)	Cell body	+++	+++	++	+	+++
	Cell processes	+	+++	++	-	++
Premyelinating OLs (O4+)	Cell body	+++	+++	++	+	+++
	Cell processes	+	+++	++	-	++
Mature OLs (MBP+)	Cell body	+++	+++	++	+	++
	Cell processes	+	+++	++	-	+

2. We are testing functionality of these receptors via live-cell calcium imaging of primary oligodendrocytes in culture to assess their response to broad spectrum muscarinic receptor agonists, Acetylcholine and Carbachol, via a rapid Calcium influx, indicating that the receptors on these cells are functional.

Future work will include characterizing the function of muscarinic receptors on oligodendrocyte biology and development, so as to provide a framework for future studies with GW agents. Once we have identified the expression of cholinergic receptors on oligodendrocytes we will determine their function in biological processes that could account for white matter injury seen in GWI. This includes effects on cell proliferation, survival, differentiation, formation and maintenance of myelin. Bioassays for all of these functional effects will be undertaken. After determining the cellular effects in culture (e.g., proliferation, survival, differentiation, migration, myelination, demyelination) this information will inform us how best to undertake animal studies and the best strategy to measure the effects. Our immediate goals are to determine how disrupting Ach signaling (and neuroinflammation/cytokines) could cause the white matter deficits seen in GWI. There are two general ways that this could happen: 1) Direct effects on oligodendroglia, 2) Alternatively, by disrupting cholinergic signaling from axons that affects oligodendrocytes and myelination. The use of experiments on OPCs in monoculture vs co-culture with neurons will enable us to make this critical distinction.

Researchers have discovered that release of synaptic vesicles from perinodal astrocytes remodels myelin structure and exposes the node of Ranvier to disruption. This is relevant to immune attack on myelin and would be one of the consequences of GFAP upregulation that accompanies TBI and neurotoxicity. This has led to the identification of a possible biomarker for myelin damage (neurofascin 155) that the GWIC investigators will now investigate in CSF and serum samples of veterans with GWI from the consortium biorepository as part of a newly funded study in collaboration with Dr. Sullivan, Klimas and Krengel (GW140140). Dr. Fields is developing methods with high sensitivity to detect this protein fragment (ELISA methods) for further study and progress to date is listed below.

1. Dr. Fields lab has developed a custom Sandwich-ELISA that can potentially detect cleaved fragments of Neurofascin 155. The lab has received the custom antibodies and are beginning to characterize the efficacy of the ELISA system.

2. Future work will include testing the NF155 ELISA kit for efficacy and if everything works out, we can use the assay to look for cleaved NF155 fragments in CSF and serum samples of veterans with GWI (from the GW repository in the newly funded study).

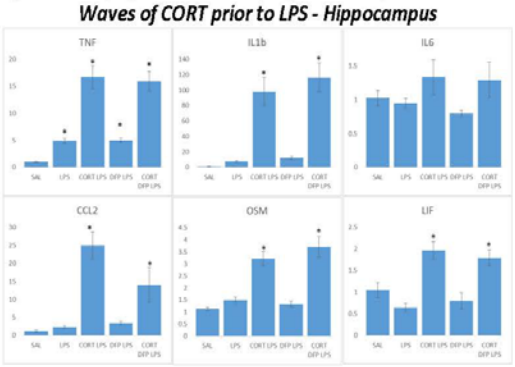
Task 4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the chronic nature of GWI (CDC – 100 C57BL/6 mice).

An LPS challenge at 21 days post exposure to CORT and CORT + DFP and following another 4 days of CORT exposure produces enhanced neuroinflammation, and some markers trend toward greater neuroinflammatory levels for mice initially exposed to CORT + DFP. CDC investigators performed cytokine expression profiling in the SD rat after DFP and CORT+DFP and determined that the enhanced cytokine expression seen in the GWI mouse model could also be generalized to the rat strain used in Dr. Maier's studies at the University of Colorado. Specific mouse model studies have also begun and results are presented in figures below for 20 day and 90 day post-exposure periods. In Figure 7 below, an LPS challenge at 21 days post exposure to CORT and CORT + DFP and following another 4 days of CORT exposure produces enhanced neuroinflammation, and some markers trend toward greater neuroinflammatory levels for mice initially exposed to CORT + DFP. These results suggest that neuroinflammatory does indeed occur chronically with GW-relevant neurotoxicants (DFP + CORT) in the chronic period as well as the acute time period.

21 Days after DFP Exposure and Successive Waves of CORT prior to LPS - Grouping

Group	*N per group*
Non-handled Saline	5
Saline LPS	5
CORT LPS	5
DFP LPS	5
CORT DFP LPS	6

Neuroinflammatory Cytokines -21 Days after DFP Exposure and Successive Waves of CORT prior to LPS - Hippocampus



90 Days after DFP Exposure and Successive Waves of CORT prior to LPS

Group	Time point	n
CORT SAL SAL	90 day	5
CORT SAL LPS	90 day	6
CORT DFP SAL	90 day	6
CORT DFP LPS	90 day	5
Group	**Time point**	**n**
WTR SAL SAL	90 day	5
WTR SAL LPS	90 day	5
WTR DFP SAL	90 day	5
WTR DFP LPS	90 day	5

97 Days after DFP Exposure and Successive Waves of CORT prior to LPS

Group	Time point	n
CORT SAL LPS	12 week	5
CORT DFP SAL	12 week	5
CORT DFP LPS	12 week	5

Neuroinflammatory Cytokines – 90 days or 12 weeks after DFP exposure and successive waves of CORT - CORTEX

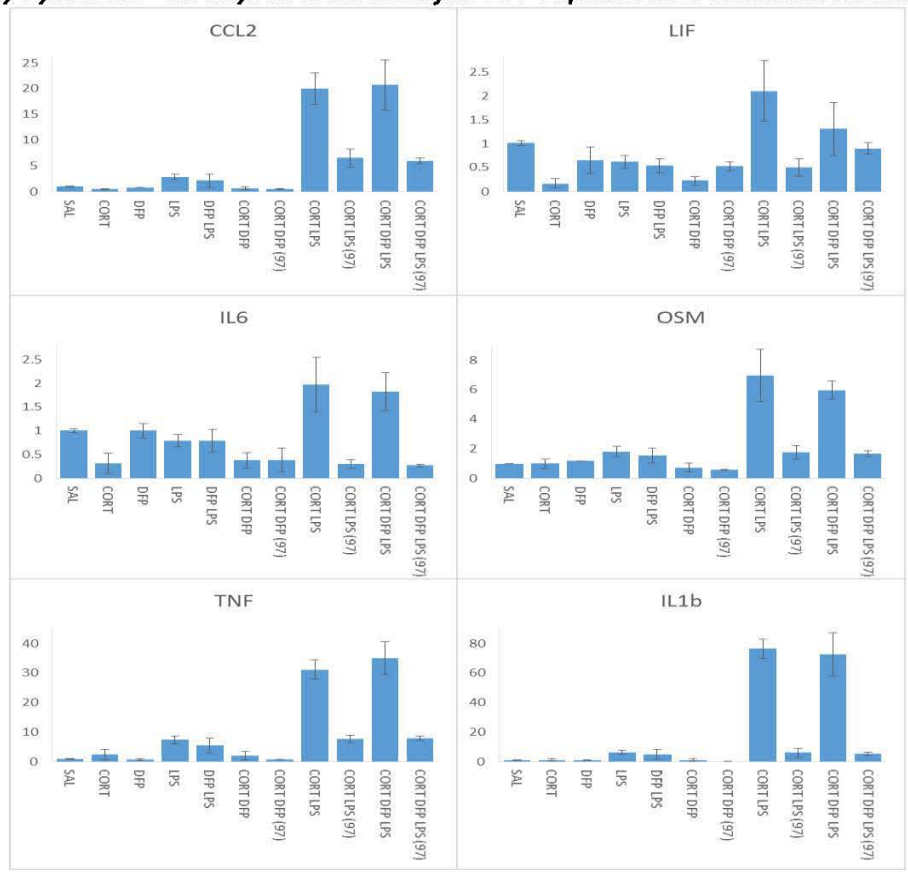
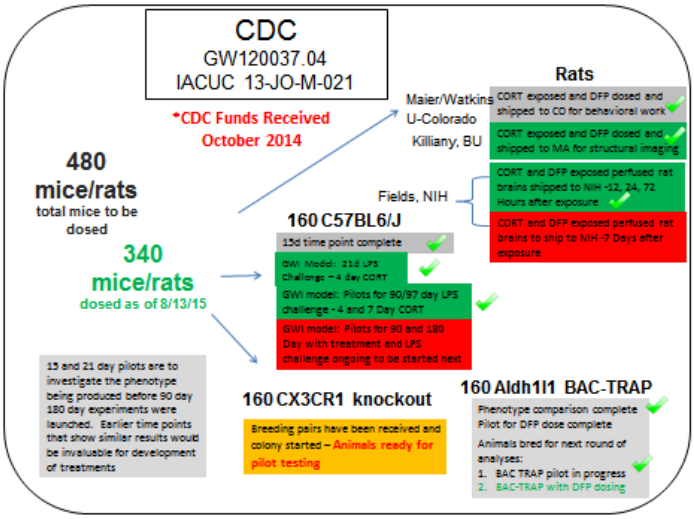


Figure 8*. An LPS challenge at 90 days post exposure to CORT and CORT + DFP, with exposure to successive waves of CORT (4 Days ON and 10 Days off, every two weeks) produces enhanced neuroinflammation, and some markers trend toward greater neuroinflammatory levels for mice initially exposed to CORT + DFP. Additionally, CORT exposure leading up to the LPS challenge is necessary for inducing enhanced neuroinflammation as neuroinflammatory results are not seen when LPS is administered during the CORT off period.*

Task 4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate "drugable" targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129-Cx3CR1 mice).

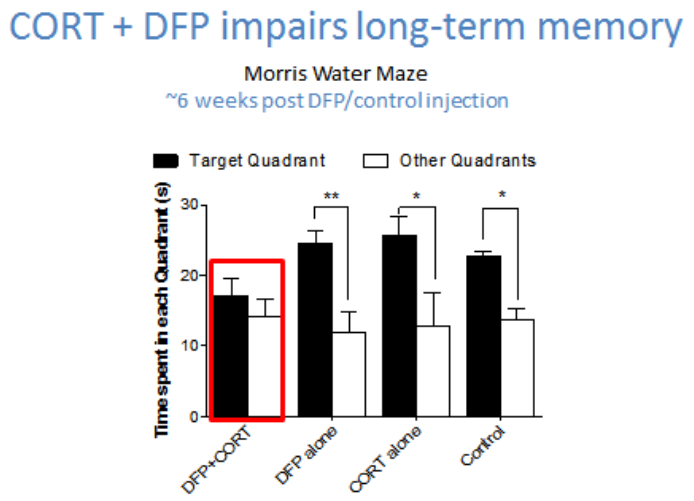
- CDC CX3CR1 knockout breeding colony has produced enough mice for pilot testing of the CORT DFP model to see if this produces enhanced neuroinflammation in these mice. The CDC has plans for this type of testing in November 2015. Data gleaned from work with CX3CR1 KO mice and the GWI exposure model, will allow us to better answer how microglia contributes to our phenotype. If microglia are critically important for development of GWI phenotype, we will go on to select a drug treatment that will target microglia cells and attempt to reduce GWI symptoms by targeting these cells.



- ALDH1L1 BAC-TRAP mice have been used for initial studies with the CDC GWI exposure protocol, and we see that DFP exposure produces a similar phenotype in these animals to that seen in C57 exposed animals. The TRAP procedure was then used to isolate actively translating mRNA from astrocytes (ALDH1L1-containing cells) at 6 hours after DFP exposure with and without CORT. Analysis of this data is ongoing.

Task 4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado – 120 rats).

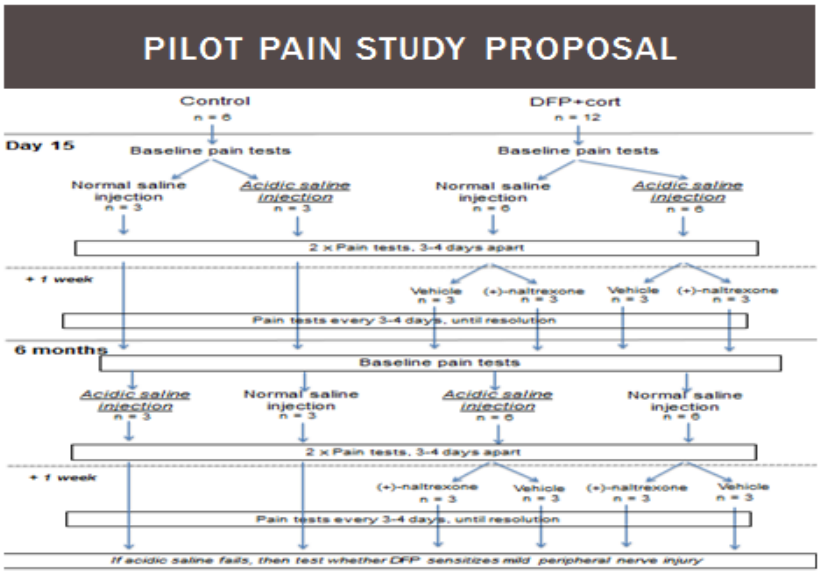
The purpose of the proposed research was to determine whether the combination of exposure to corticosterone (CORT; mimicking "stress") and DFP (mimicking sarin organophosphate exposure) produces cognitive deficits in rats and whether any such deficits are hippocampal in origin. To this end, Dr. O'Callaghan prepared 4 groups of rats for an initial study—Control, DFP, CORT, and DFP + CORT. For this initial study 6 rats were included in each group, and were shipped to University of Colorado after dosing at CDC. These rats were tested for the formation of spatial memory (hippocampal dependent) using the Morris water maze and for contextual fear memory after fear conditioning. Anxiety was also assessed as indicated by juvenile social interaction (JSE) ratings. The water maze data were striking. Acquisition to escape by finding the safe platform in the spatial version of this task was severely impaired in the DFP + CORT group. However, escape was acquired. Dr. Maier then tested for memory of where the escape platform is located by testing the animals 24 hrs later with the escape platform absent. If the subject remembers they are expected to spend their time in the quadrant where the platform had been located, the target quadrant was Q2, and all the groups except the DFP + CORT group spent much more time in this quadrant than would be expected by chance. That is, they remembered where the platform was located. The DFP + CORT group showed worse memory performance and spent almost equal time in each quadrant (see Figure). The fear conditioning data were less clear and more subjects are needed in further studies to make more definite conclusions. These data are highly encouraging and larger group sizes will make results and conclusions more evident. One difficulty encountered was that JSE testing suggested that all animal groups were anxious. This may have been related to the fact that the animals were shipped from Dr. O'Callaghan's laboratory after dosing treatment, and so discussions are now ongoing to determine whether the "dosing" should be done directly in Colorado under Dr. O'Callaghan training rather than at the CDC laboratory for future planned studies.



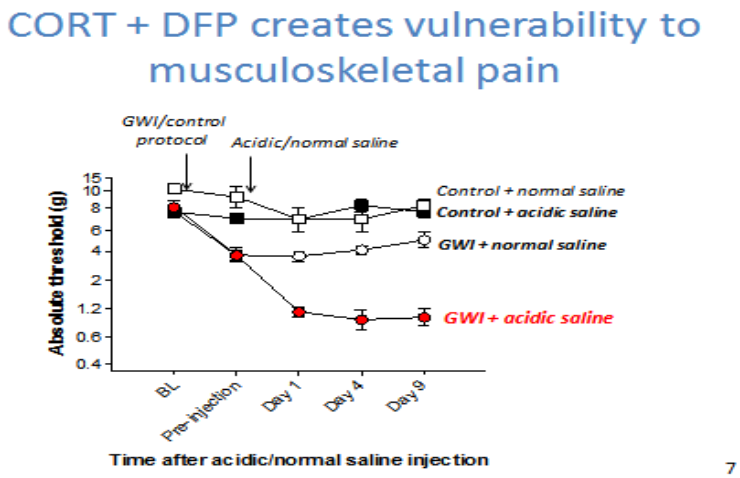
Initial pain studies were planned for the behavioral studies at the University of Colorado but the initial animal testing indicated that the same animals could not be used for the memory and pain testing studies. Therefore, Dr. Watkins submitted a proposal for pain assessment and the first pilot treatment study in a rat model of +naltrexone in the GWI consortium. This proposal was approved by the GWIC steering committee and the GOR to use a small amount of exploratory funds to begin the pain studies. This study will integrate chronic

pain modeling into our animal studies and will start our pilot treatment studies. Dr. Watkins' group will assess for chronic pain in the GWI animal model and will then try the +naltrexone pain treatment in the animals. Specific pilot study methods and figure are described below:

Rats will be randomized to either GWI (n = 12) or control (n = 6) groups. Fifteen days after induction of the GWI model, baseline pain tests (mechanical allodynia (Milligan et al., 2001) and thermal hyperalgesia (Hutchinson et al., 2008) will be performed and rats will receive intramuscular injections of either normal (pH 7) or acidic saline (pH 4) (GWI: n=6/group; control n=3/group). Pain tests will be performed every 3-4 days for a week. The TLR4 antagonist (+)-naltrexone (n=3/group) or vehicle control (n=3) will be administered for 3 days (18 mg/kg/day; SC) to the GWI group. Pain tests will be performed every 3-4 days until thresholds return to baseline levels. Six months after induction of the GWI model, baseline pain tests will be performed and rats will receive intramuscular injections of either normal (pH 7) or acidic saline (pH 4) in a cross-over design. Pain tests will be performed every 3-4 days for a week. The TLR4 antagonist (+)-naltrexone (n=3/group) or vehicle control (n=3) will be administered for 3 days (18 mg/kg/day; SC) to the GWI group, in a cross-over design. Pain tests will be performed every 3-4 days until thresholds return to baseline levels (see pain study figure below).



This study has been approved by the University of Colorado-Boulder IACUC, assuming receipt of prepared rats for the study or decision to prepare rats at U-Colorado now that approval has been obtained to dose onsite. A small initial pilot study was performed to assess whether CORT + DFP model would work for inducing musculoskeletal pain in the rat model. Results showed that absolute pain thresholds were greatly decreased after CORT + DFP and then acidic saline. The pilot pain study will continue to assess how long the reduced pain threshold will last in the model and whether the pain will be reversible with a glial inhibitor (+naltrexone) or how long the CORT+DFP sensitization will last.



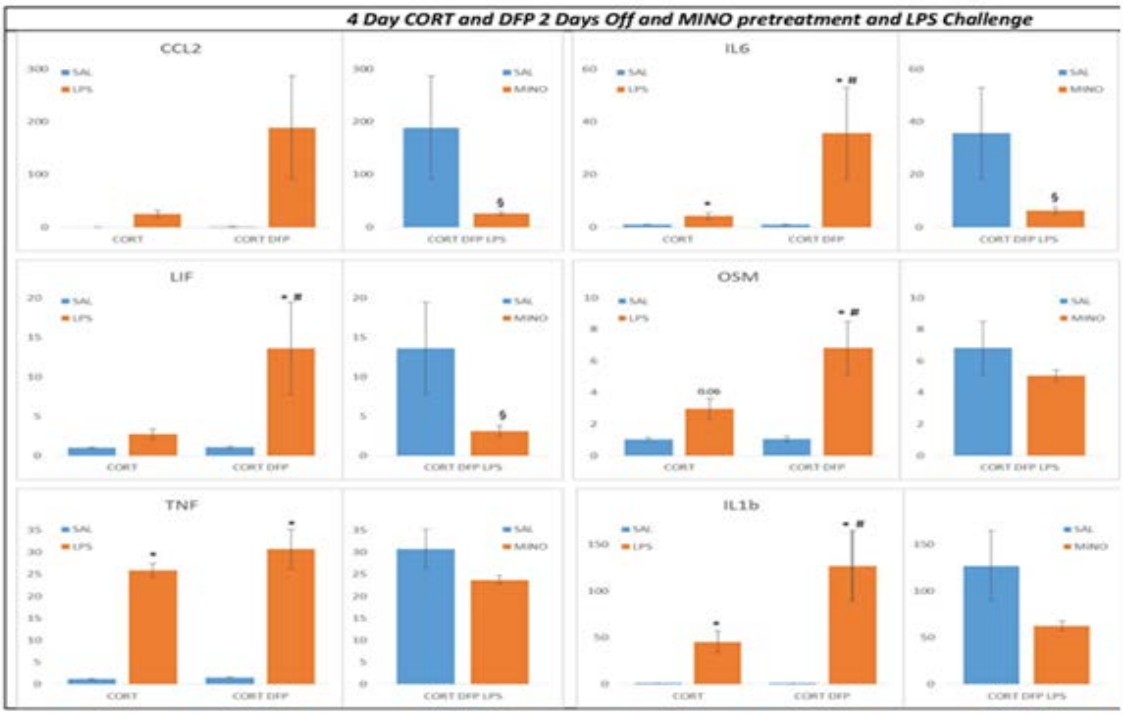
Task 4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from GWI neuroinflammatory rodent models (CDC – 60 rats, Nova).

This study plans to compare blood samples and brain tissue proteins from 60 dosed mice. The animals were dosed and sacrificed and the blood samples were sent to NOVA university for analysis of proinflammatory cytokine/chemokine panels. Analyses have been completed and sent back to Dr. O'Callaghan's laboratory to be compared with brain tissue studies. Initial results did not show strong markers of peripheral inflammation in comparison to central markers of inflammation. Current results are presented below.

Current progress: Liver and serum samples were obtained from the GW model 15 day cohort at CDC. Liver mRNA expression (CDC) and serum protein concentration (NOVA) was compared to mRNA brain data from the CDC for mice in the GW model: control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5). While CORT pretreatment exacerbated DFP-induced neuroinflammation, in the periphery the opposite effect occurred with CORT pretreatment ameliorating DFP-induced proinflammatory cytokine expression in the liver and serum. The divergence of expression of pro-inflammatory cytokines seen in brain and in the periphery suggests that the initial symptoms of GWI may be a result of the effects of CORT and DFP in the brain only. These findings are in an in-progress manuscript from the CDC.

Task 4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado)(20 animals per treatment).

These important experiments will begin soon when more information is known from the initial pathobiology studies in order to use those results to target appropriate choices for study. As previously mentioned, the first pilot treatment study of +naltrexone will be started shortly by Dr. Watkins at U-Colorado and initial studies using minocycline, an anti-inflammatory antibiotic has also been started by Dr. O'Callaghan's lab at CDC. Initial results are presented in the figure below.



4 Day CORT and DFP 2 Days Off and MINO pretreatment and LPS Challenge

Group	Day 5 Dosing	Day 7 Pre Treatment	Day 7 Treatment	n
CORT	SAL	-	SAL	5
CORT	SAL	-	LPS	5
CORT	DFP	-	SAL	2
CORT	DFP	-	LPS	3
CORT	DFP	MINO	LPS	5
Water	DFP	-		5
Water	DFP	LPS		5

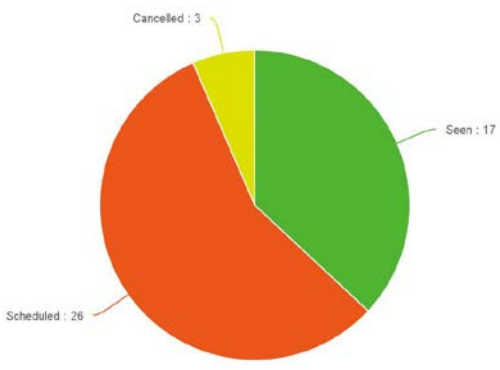
TASK 5. SCREENING, RECRUITMENT AND ASSESSMENT OF GULF WAR VETERANS FROM THREE SITES (MONTHS 9-42)

Participant recruitment and screening has begun at the Boston and Miami sites. Currently 47 participants have been screened and 26 are currently scheduled at the Boston and Miami sites. The Central Texas site will not begin recruitment until the next year to coincide with the longitudinal design of the Texas site of the consortium study. See current recruitment table below. Although below initial estimates, recruitment is steadily increasing and retention is excellent from time of screening to completion of study (see figures below)

GWIC: Gulf War Illness Consortium
Screening and Enrollment Summary Report
10/16/2015

	Total	Boston	Miami	Texas
Number of Subjects Contacted	56	40	16	0
Number of Subjects Screened	47 (83.9%)	31 (77.5%)	16 (100.0%)	0 (.%)
Number of Subjects Eligible	39 (83.0%)	24 (77.4%)	15 (93.8%)	0 (.%)
Number of Subjects with Appointments Made	26 (66.7%)	16 (66.7%)	10 (66.7%)	0 (.%)
Number of Subjects Assessed	17 (65.4%)	10 (62.5%)	7 (70.0%)	0 (.%)

	Total		Boston		Miami		Texas	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	35	12	21	10	14	2	0	0
Number of Subjects Eligible	29	10	16	8	13	2	0	0
Number of Subjects Assessed	13	4	7	3	6	1	0	0



Task 5a. Obtain informed consent from potentially eligible GW veterans

Recruitment has been ongoing for several months and 56 total subjects have contacted GWIC coordinators through print advertising, free newsletters to VSOs or social media outlets. From these contacts, 47 were found eligible to participate in the studies. Those not able to participate were largely not GW veterans. Of this group, 26 have been scheduled and 17 have completed the study protocols at Boston or Miami sites. Those participants who screen in have largely completed the studies (see figures). We expect to increase recruitment to catch up to the expected recruitment goals in the coming months.

Task 5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva samples.

26 study participants have been recruited to date and subject demographics and self-reported symptoms are reported below for the 17 study completers (13 GWI, 4 controls). As planned, cognitive assessment data will be analyzed with neuroimaging data to assess for brain-behavior relationships in GWI and will be presented below for the initial sample of study participants. These first participants allowed us to solidify and enhance all recruitment, shipping and sample sharing procedures for the clinical studies. Initial demographic outcomes suggest a fairly diverse cohort of veterans in terms of race and gender as well as self-reported neurotoxicant exposure during the war which will be helpful with planned gene-exposure and SNP analyses.

Table 4. Subject Demographics N= 17

	Mean or %	Min.	Max.
Age at time of study	**51**	**42**	**64**
Years of education	**14.4**	**12**	**18**
Female	**16%**		
Ethnicity			
Caucasian	**71%**		
African American	**24%**		
Other	**5%**		
GW-related Exposures			
Reported PB pill usage	**65%**		
Wore pesticide treated uniforms	**41%**		
Used pesticide cream or sprays	**65%**		
Chemical/biological weapon exposed	**43%**		
MFI-20 Fatigue Score (sd)	**66.6 (20.8)**		
McGill Pain Score (sd)	**26.9 (19.5)**		

Table 5 shows the percentage of participants reporting mild traumatic brain injury (TBI) before, during or after the Gulf War. These results suggest that half of our cohort to date report sustaining a mild TBI during the Gulf War. Although it would not have been intuitive that GW veterans may show increased rates of TBI due to the short duration of the conflict, these questions were added to the study protocol after rates of mTBI were found to be much higher than expected in the large longitudinally followed Ft. Devens Cohort resurvey study conducted by Drs. Krengel, Janulewicz-Lloyd and Sullivan in a separate study (Yee et al., 2015). In this study, Drs. Krengel, Sullivan and Janulewicz-Lloyd reported that GW veterans reporting a history of TBI had a significantly greater risk of reporting many chronic health symptoms (in multiple domains outside of the CNS) and with meeting criteria for chronic multisymptom illness criteria (Fukuda et al., 1998). Thus it was hypothesized that mTBI in addition to GWI neurotoxicant exposures could have been resulted in a chronic neuroinflammatory state in these veterans. Future concurrent studies with the GWIC and the Ft. Devens cohort will further assess this possible multiple-hit hypothesis of GWI in subgroups of veterans and whether the chronic sequelae is worse in these groups. This pattern also fits with recent work reported by other investigators who coined the term post-concussive syndrome (PCS) or post-inflammatory brain syndromes (PIBS) that result after TBI, neurotoxicant exposures (chemotherapy, nerve agents), post-operatively etc. (Rathbone et al., 2015). PIBS/PCS result in chronic neuroinflammatory signaling of cytokines (IL-1b, IL-6, TNF-a) and have been associated with many behavioral effects including cognitive problems, headaches, fatigue, mood alterations and sleep problems (Rathbone et al., 2015). Initial results are presented below of behavioral correlation of cytokines with cognitive, health symptom and neuroimaging outcomes collected to date. Tables 6 and 7 describe the neuropsychological test battery and clinical study surveys administered to study participants.

Table 5. mTBI reporting

mTBI self-report	GWIC Cohort	Ft. Devens Cohort
% Pre-War	**38**	**44**
% During GW	**50**	**31**
% Post-War	**25**	**44**

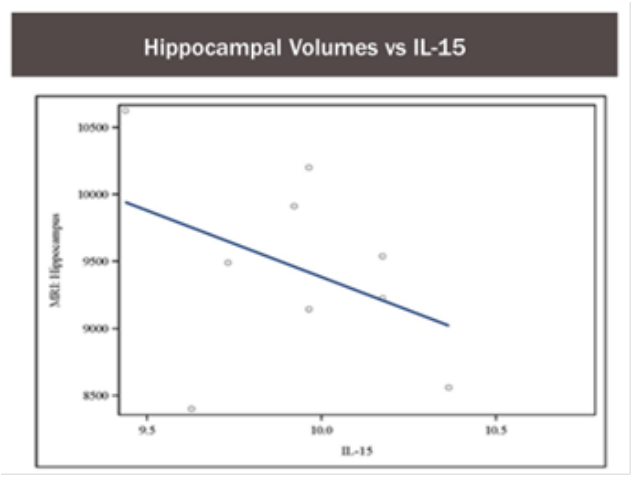
Table 6. Neuropsychological Test Battery for GWI Consortium Study

Test Name	Description	Outcome Measure
I. Executive System Functioning		
Controlled Oral Word Association Test (COWAT)	Spontaneous generation of words from letters F, A and S and animals category.	Total correct words generated
Stroop Test (DKEFS)	Timed response requiring naming of ink color and inhibiting discordant color-names; measures fronto-executive, selective response and inhibition.	Total Errors
II. Tests of Attention, Vigilance and Tracking		
Trail-making Test (Reitan &Wolfson, 1985)	Timed connect-a-dot task to assess attention and motor control requiring sequencing (A) and alternating sequences (B).	Time to Completion
Continuous Performance Test (Connors')	Target letter embedded in series of distractors; to assess sustained attention and reaction time.	Reaction Time, Total Omission and Commission Errors
III. Tests of Motor Function		
Grooved Pegboard Test (Klove, 1963)	Speed of inserting pegs into slots using each hand separately; assesses motor coordination and speed.	Raw Score time to completion
Finger Tap Test (manual tapper)	Continuous tapping of computer key with alternate hands; assesses simple motor speed.	Number of taps
IV. Tests of Visuospatial Function		
Block Design Test (WAIS-IV)	Copy picture designs with blocks	Raw Score
Rey-Osterrieth Complex Figure Test	Copy of a complex figure	Total correct out of 36
V. Tests of Memory		
California Verbal Learning Test (CVLT-II; Delis et al., 2000)	List of 16 nouns from 4 categories presented over multiple learning trials with recall after interference; assesses memory and learning strategies.	Total Trials 1-5 Long Delay
Rey-Osterrieth Complex Figure Test	Immediate and delayed recall of a complex figure	Total recall out of 36
VI. Test of Motivation		
Test of Memory Malingering (TOMM)	Test of Memory Malingering (TOMM) is a 50-item visual recognition test designed to help distinguish malingering from genuine memory impairments	Total correct
VII. Mood measure		
Profile of Mood States	65 single-word descriptors of affective symptoms summed on six mood scales.	T – Scores

Table 7. Gulf War Illness Consortium - Survey and Questionnaire Instrument Descriptions

Name	Description
Demographics	Subjects report information on age, education, gender, ethnicity, marital status, GW duty service (active vs. reserve/National Guard), military rank and current military status.
SF36V	Veterans' version of the SF36 which assesses functional health-related quality of life in 8 domains and provides overall summary scores for physical and mental health status.
Kansas Gulf War and Health Questionnaire	Queries veterans about demographics, military and deployment history, and chronic symptoms and diagnoses required to ascertain Kansas GWI and CMI case status.
Medical Conditions	A checklist with 21 medical conditions that the subject is asked to rate if they have ever had the condition, how it was diagnosed (self or doctor) and when it was diagnosed.
Kansas Gulf War Experiences and Exposures	A questionnaire that assesses veteran-reported experiences and exposures during their deployment to the 1991 Gulf War.
Structured Neurotoxicant Assessment Checklist (SNAC)	The SNAC assesses the degree of past and current exposure to neurotoxicants during civilian and military occupations and includes questions pertaining to recent occupational and environmental exposures.
Pittsburg Sleep Quality Index (PSQI)	PSQI assesses sleep quality during the past month. It covers domains of sleep quality, latency, duration, efficiency, disturbances, medications and daytime dysfunction. Total global scores range from 0-21.

Task 5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis. MRI scans were obtained from the first 9 study participants at the Boston site. When the Baylor site begins recruitment, MRI scans will be transferred electronically in either extended DICOM or par/rec format to the Center for Biomedical Imaging at Boston University School of Medicine. Each scan undergoes quality checking that consists of a visual inspection for the presence of noise or artifact as well as a review of scan parameters to ensure that the appropriate ones were used in the acquisition. Scans that fail the quality check are rejected by the study and remediation discussed with the site investigator. Scans that pass the quality check enter the post-processing pipeline. The first 9 scans have been through the post-processing pipeline and initial correlation results have been run. Correlation analyses of hippocampal volume and IL-15 suggest a negative correlation of brain volume and cytokine levels in this first round of correlation analyses. Further analyses will be run with the larger study sample as they are available.



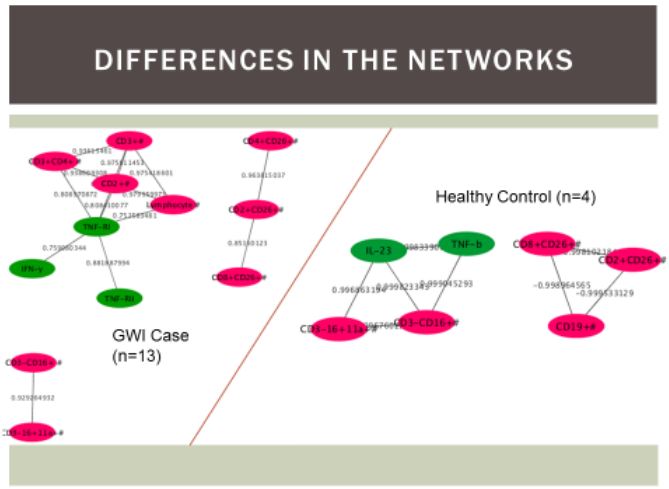
MRI Imaging: The scanning session include: 1) Three plane TFSE scout scan, 2) a Sense reference Scan, 3) an accelerated high resolution MPRAGE scan acquired in the sagittal plane, 4) a multi-component T2 imaging sequence acquired in the axial plane, 5) a Diffusion Tensor Scan with 32 directions acquired in the axial plane, 6) a resting state functional magnetic resonance imaging scan, and 7) a pCASL sequence obtained while the participant is at rest and 8) a High Angular Resolution Diffusion Imaging (HARDI DTI) scan.

Task 5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.

Data from the first 17 participants has been scored and cleaned and some interesting correlation analyses are presented in the figures below. As study protocols are obtained and data is collected, quality control procedures will remain in place including double entry of data collection forms in the REDCap data collection website, built in range checks and quality control audits of all data collection by the Data Coordinating Center staff and the local BU Administrative Core neuropsychologists. Dr. Toomey also conducts bimonthly conference calls to review scoring and quality control, as well as regular reviews of data entered and spot checks of any questionable data to ensure data administration and scoring integrity throughout the recruitment period. This will ensure the highest quality data available for analysis.

Task 5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.

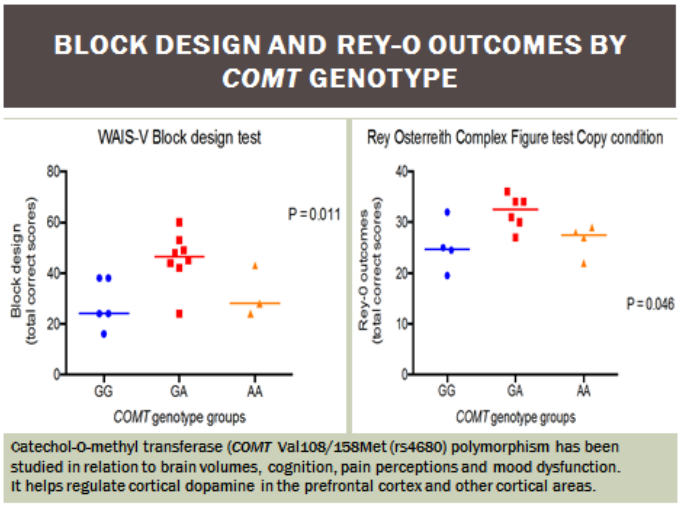
Blood and saliva samples have been sent to NOVA Southeastern University for each of the recently completed 17 study participants. The analysis of 16 cytokine and chemokines has been completed for the first batch of samples and a representative network analysis was run for the first group of cases (n=13) and controls (n=4) suggesting important early differences in correlation patterns and cellular communication network connections between leukocytes (red) and cytokines (green). Additional preliminary correlation analyses results are shown in the sections below. Cortisol measurements that will include testing for neuroendocrine and immune alterations and for hypothalamic pituitary adrenal axis abnormalities will occur in larger batch samples. Specifically, blood samples are being sent to NOVA Southeastern University for analysis of proinflammatory cytokine and chemokines, monocyte markers (MCP-1), and nanostring analysis of mRNA and miRNA of proteins related to TLR4 functioning and glial activation including miR-155, miR-21 and miR-146. Multiplex Quansys ELISA system is being used with an existing cytokine platform created by Dr. Klimas' research laboratory. Dr. Klimas is measuring 16 cytokines in plasma. Gene expression and pathways will also be assessed using an Agilent microarray system and quantitative real-time PCR for validation of differentially expressed genes. Preliminary results of correlation analyses of select cytokines with behavioral and neuroimaging outcomes are shown below.



Task 5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis

The first batch of 17 saliva samples have been sent to the University of Adelaide for genetic polymorphism studies multiple genetic variants of important immune mediators and immune receptors involved in glial activation and how these genetic polymorphisms alter pathologies of GWI will be assessed. Variability in immune genetics targets of interest such as the gene encoding for IL-1B will be determined in the GWI populations and via comparison with a healthy control population will be related to both the development and severity of GWI symptoms when larger sample sizes are available. Results of behavioral and neuroimaging outcomes in relation to IL-1b are presented below and will be correlated with genetic polymorphism data when it is obtained.

In this first batch of 17 samples, genomic DNA was isolated from the saliva samples and genotyped for targets of interest with custom-designed multiplex analysis in Dr. Coller's lab. The samples were analyzed for one initial



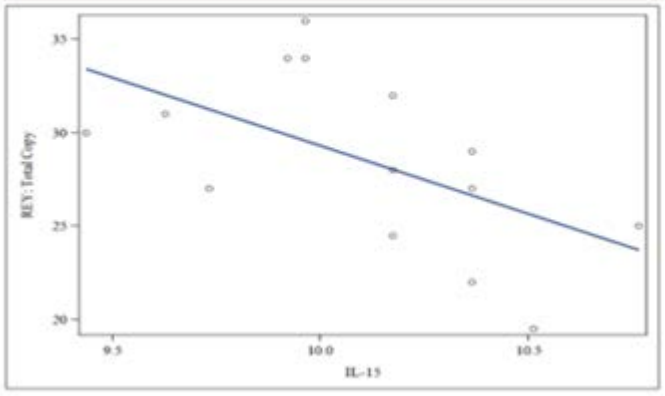
immune genetic markers of interest; Catechol-O-methyl transferase (COMT). COMT is associated with synaptic catecholamine neurotransmitters. COMT helps regulate cortical dopamine in the prefrontal cortex and other cortical areas (Tunbridge et al, 2006). COMT val108/158met polymorphisms have been studied in relation to brain volumes, cognitive functioning, pain perceptions and mood dysfunction. The AA MET variant is usually associated with more dopamine and GG VAL with less dopamine. Both high and low DA can be related to worse cognition. In this first analysis of genetic polymorphisms of COMT, results indicated that the COMT GA variant was significantly associated with better performance on two measures of visuospatial functioning including the WAIS-V Block Design test (Kruskall-wallis statistic 7.6; p=01) and the Rey Osterreith Complex Figure test Copy condition (Kruskall-wallis statistic 5.8; p=05). These findings of two tests of visuospatial functioning being significantly correlated with COMT genetic polymorphisms could suggest a vulnerability of some veterans to worse visuospatial functioning that will be further assessed when larger sample sizes are available and will be added to the cognitive outcome risk factor analyses going forward.

Task 5g. Conduct preliminary analyses of clinical data

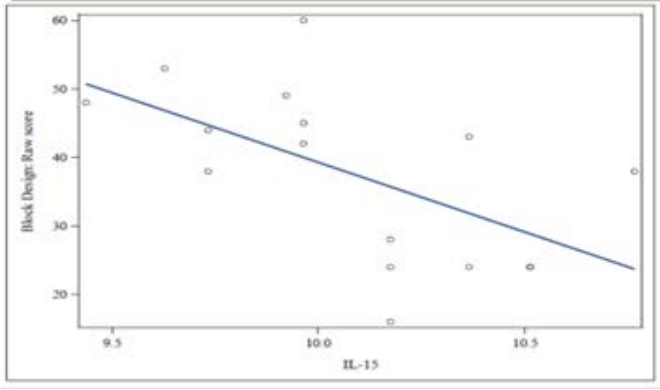
The BUSPH Data Coordinating Center has cleaned all current data and prepared the datasets for statistical analysis from the REDCap data capture web database in direct collaboration with the study biostatistician Dr. Heeren, Dr. Sullivan and the study PIs. Results of initial Pearson correlation coefficient analyses of cognitive testing outcomes with neuroimaging data (in this quite small sample size) suggest some interesting potential relationships in structure-function relationships of brain-behavior-immune interaction outcomes. An example includes correlations with hippocampal volumes and making more errors of commission (hitting the wrong target by not recalling the task specifications) on the computerized continuous performance test 3 (Connors CPT). Other examples include the visual memory task, the Rey-Osterrieth Complex Figure task appearing mildly correlated with hippocampal volume would be expected and was shown in our prior studies (Sullivan et al., 2013). Future analyses in the larger study samples will be adjusted for common covariates including age, education and gender.

The Figures below show Pearson correlation coefficient analyses of cytokine and symptom reporting and cognitive outcomes that suggest potential important immune-behavior outcomes particularly with respect to IL-1b and IL-10 with pain reporting on the VAS pain scales and the McGill Pain Questionnaire that will be further assessed when larger sample sizes are available. In terms of cognitive outcomes, examples of correlations found include CPT-3 reaction time score appearing to be highly correlated with IL-1a and two measures of visuospatial functioning (Rey-Osterreith Complex figure Copy Condition and Block Design Test) being inversely correlated with IL-15 measures. These relationships will be further compared when larger sample sizes are available to assess by case-control status. Although IL-15 has recently been reported to be associated with fatigue ratings, we did not find this correlation in this small initial study sample with IL-15 although a promising correlation was found for IL-10 with the fatigue ratings on the multi-dimensional fatigue inventory (MFI-20) perhaps suggesting a compensatory mechanism with increased IL-10 levels due it its anti-inflammatory properties (Parkitny, Younger et al., 2015). All results will be compared when larger study samples are available and less speculative conclusions can be drawn.

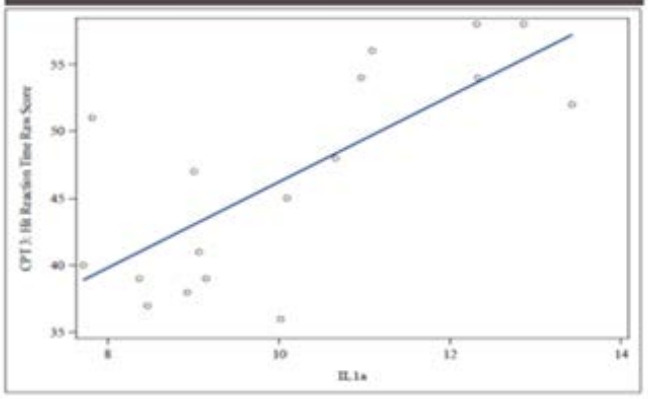
REY-O COPY VS. IL-15



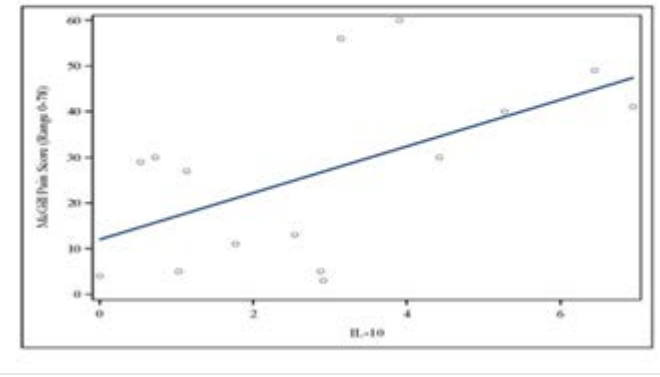
BLOCK DESIGN VS. IL-15



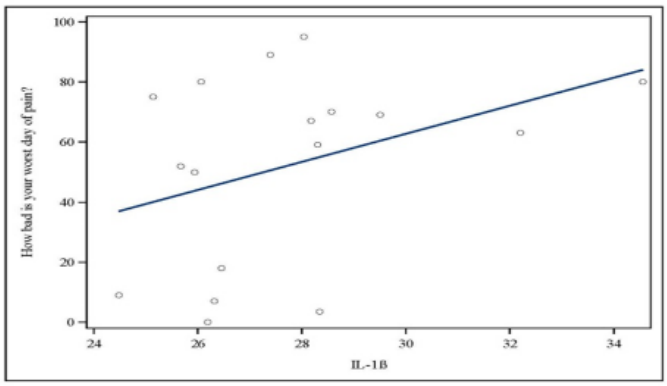
CPT REACTION TIME VS IL-1A



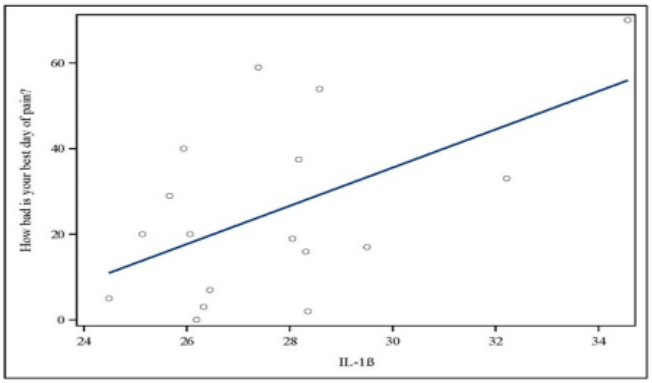
MCGILL PAIN SCALE VS. IL-10



VAS WORST PAIN SCALE VS. IL1-B



VAS BEST PAIN SCALE VS. IL-1B



KEY RESEARCH ACCOMPLISHMENTS

- Obtained final IRB and HRPO approvals for Nova Southeastern University, Miami VA and Boston University. Obtained Exempt status at University of Adelaide.

- Trained all clinical staff in-person on neuropsychological testing and structured clinical interviews and obtained all necessary equipment for three clinical sites.

- Data Coordinating Center has programmed online surveys in REDCap web data capture software, developed a bar-coded specimen tracking system and a consortium website for subject recruitment.

- Subject recruitment for clinical studies has begun and 26 study participants are currently scheduled.

- Initial clinical studies have begun and are showing promising results for:

    - Brain-behavior relationships in study cohort as shown by structure – function correlations
    - Brain-immune relationships as shown by cognitive outcomes – cytokines correlations
    - Immune-behavior relationships as shown by symptom reporting – cytokine correlations
    - Immune-structure relationships as shown by MRI volumes – cytokine correlations
    - Building a Neuroinflammatory Risk Profile of GWI based on early study results

- Obtained final protocol approvals by the respective IACUCs and ACURO for the preclinical animal research sites at CDC/NIOSH, NIH, Temple University and University of Colorado.

- Initial preclinical studies have begun and are showing promise results for:
    - Behavioral effects on memory and pain thresholds of the DFP + CORT neuroinflammatory model of GWI in rats.
    - For axonal transport alterations in the in-vitro GWI model that show CORT exacerbates DFP.
    - For altered myelination (increased MBP) and glial (astrocyte) signaling in the GWI model with a new potential biomarker identified that will be targeted for further study (NF-155) in a newly funded grant.
    - Chronic neuroinflammatory phenotype shown in later time points in mouse and rat models.

CONCLUSIONS

This multi-institutional collaboration of highly qualified GWI researchers from public universities, federal agencies, and the private sector, provide an unprecedented opportunity to more fully elucidate the underlying pathobiology of Gulf War illness in one integrated model that once proven, will lead to focused treatment trials that can be quickly implemented.

The central hypothesis for the pathobiological mechanisms of GWI in this consortium includes chronic neuroinflammation as a result of initial glial activation and then priming of glial responses that cause stronger and longer responses that do not shut off the chemical cascade of proinflammatory cytokines and chemokines that cross-talk between the immune system and the brain. This could result in a lasting multisystem illness affecting many body systems, as seen in GWI.

Improved understanding of the role of glial activation in chronic pain states has given rise to rapidly expanding efforts to identify pharmaceuticals that specifically focus on glial functions. The growing availability of treatments of this type gives particular urgency to our efforts to determine the extent to which glial activation and central cytokine activation explain the symptoms of GWI. In order to specifically address the research gaps outlined by the IOM and the RAC reports with regard to biomarker identification and pathobiology of GWI, this research team is characterizing disease symptoms and validating and improving pathobiological markers based on collective prior clinical and preclinical studies and leveraging longitudinal cohorts and stored blood samples with the ultimate goal of identifying targeted and effective treatments for GWI. Initial preliminary results suggest that the consortium animal model of GWI is correlated with behavioral alterations seen in clinical studies including altered visuospatial memory functioning and reduced pain thresholds and that chronic neuroinflammation is present in the DFP + CORT GWI model. Myelin studies show an increase in myelin basic protein at early time points with the DFP + CORT model that will be assessed at later time points for persistence and mechanism. Axonal transport is altered most dramatically by the DFP + CORT exposure model. As these preclinical models are further developed, they will be correlated with clinical studies to identify markers of the illness and targets for therapeutic intervention. Early preliminary clinical study results suggest brain-immune-behavioral outcome correlations that bode well for the derivation of a neuroinflammatory risk profile of GWI, diagnostic marker development and targeted therapeutic strategies in the near future.

PUBLICATIONS, ABSTRACTS and PRESENTATIONS

Publications

O'Callaghan, J.P., Kelly, K.A., Locker, A.R., Miller, D.B., & Lasley, S.M. (2015). Corticosterone Primes the Neuroinflammatory Response to DFP in Mice: Potential Animal Model of Gulf War Illness. Journal of Neurochemistry, 133, 708-721

Yee, M., Seichepine, D., Januelwicz Lloyd P., **Sullivan, K.**, Proctor, SP & **Krengel, M**. Traumatic brain injury, health and rate of chronic multisymptom illness in veterans from the 1990-1991 Gulf War. Journal of Head Trauma Rehabilitation. Aug 19, 2015 (epub ahead of print).

Yee, M., Seichepine, D., Janulewicz Lloyd P., **Sullivan, K. & Krengel,** M. History of Pre-War Brain Injuries Influences Total Current Health Symptoms in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 8.

Seichepine, D., Yee M., Janulewicz Lloyd P., **Sullivan, K. & Krengel, M**. Frequency of Traumatic Brain Injuries in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 14.

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Abstracts

M.B. Abou-Donia**, K. Sullivan**, L. Conboy **,** E. Kokkotou**:** Serum Autoantibodies to Neural-Specific Proteins as Objective Biomarkers for Gulf War Illness. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016.

Kelly KA, **Locker AR,** Michalovicz LT, Miller DB, O'Callaghan JP: Exploration of the gulf war illness phenotype in a mouse model challenged w LPS at long term time points. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Revitsky AR, Kelly KA, Miller DB, Lasley SM, **O'Callaghan JP**: Organophosphate-induced Neuroinflammation, With and Without Corticosterone Pretreatment, Is Not Due to Acetylcholinesterase Inhibition. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Michalovicz LT, **Locker AR, Kelly KA**, Miller DB, **O'Callaghan JP:** Corticosterone priming of the neuroinflammatory response to acetylcholinesterase inhibitors results in overexpression of TLR2 and downstream targets, but not activation of the NLRP3 inflammasome. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Michalovicz LT, **Locker AR, Kelly KA,** Miller DB, **O'Callaghan JP**: Chronic corticosterone primes the brain response to select neuroinflammatory agents by overexpression of toll-like receptor 2 and S100A8: A potential role of microglia. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Locker AR, Kelly KA, Michalovicz LT, Miller DB, **O'Callaghan JP:** Corticosterone primes the neuroinflammatory responses to Gulf War Illness associated exposures: Effects of irreversible vs reversible acetylcholinesterase inhibitors. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Kelly KA, Locker AR, Michalovicz LT, Miller DB, **O'Callaghan JP:** Phenotype comparisons of ALDH1L1 BAC-TRAP mice under control and neurotoxic (MPTP) conditions. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Revitsky AR, Kelly KA, Miller DB, Lasley SM, **O'Callaghan JP**: Pyridostigmine bromide suppresses neuroinflammation induced by DFP. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Kelly KA, Revitsky AR, Miller DB, Lasley SM, **O'Callaghan JP**: Chronic glucocorticoid and nerve agent DFP exposures produce a neuroinflammatory model of Gulf War Illness without neurodegeneration. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Presentations

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 17, 2015.

Sullivan, K., Klimas, N. Committee and Panel Discussion: 'how to discussion' for GWI Biomarker Research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC, September, 2014.

O'Callaghan, J., **Sullivan, K.** Committee and Panel Discussion: 'how to discussion' for GWI animal research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC, April, 2014.

Seichepine, D., Yee M., **Janulewicz Lloyd P**., **Sullivan, K.** & **Krengel, M**. Chronicity of Health Symptoms in the Ft. Devens Cohort. International Neuropsychological Society, 42nd Annual Meeting, Seattle, WA, February 2014.

Steele, L. Committee and Panel Discussion: 'how to discussion' for GWI Case Criteria Research Advisory Committee on Gulf War Veterans' Illnesses; Winter Meeting, Washington, DC, January, 2014.

Seichepine, D., Yee, M., **Janulewicz Lloyd P., Sullivan, K.**, Proctor, S., & **Krengel, M.** Traumatic Brain Injury and Health Status of Veterans from the 1990-1991 Gulf War. Boston University Second Annual Joining Forces TBI/PTSD Event, Boston, MA, December, 11, 2013.

Sullivan, K. RAC-GWVI Treatment Development Discussion. Research Advisory Committee on Gulf War Veterans' Illnesses; Summer Meeting, Washington, DC, June, 2013.

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 26, 2013.

INVENTIONS, PATENTS AND LICENSES – None

REPORTABLE OUTCOMES

Newly Funded Studies

- **Title: An in-vivo investigation of Brain Inflammation in Gulf War Illness with Integrated PET/MR imaging (PI: Loggia)**
  Supporting agency: Department of Defense (GW130100)
  Performance period: 9/1/14-8/31/17
  Level of funding: $1,026,352
  Brief description of project's goals/ Specific aims: The overarching objective of this study is to demonstrate the pathological occurrence of microglial activation in the brains of patients with Gulf War Illness and document the effects of this activation on Gulf War Illness symptomatology and brain physiology using new imaging approaches. The project's three specific aims are (1) to demonstrate in vivo activation of microglia in veterans with Gulf War Illness, (2) to demonstrate the association between microglial activation and alterations in brain physiology and anatomy, and (3) to demonstrate an association between microglial and neural activity with symptom severity; i.e., fatigue, pain, disability, depression, and anxiety.

- **Title: Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans With Gulf War Illness (PI: Sullivan and Abou-Donia)**
  Supporting agency: Department of Defense (CDMRP/GWIRP) (GW140140)
  Performance period: 9/1/15-8/31/18
  Level of funding: $449,751
  Brief description of project's goals/ Specific aims: We hypothesize that following neural damage in GWI there is loss of cells, breakdown of the blood brain barrier leading to leakage of specific

neuronal and glial proteins into circulation, with subsequent formation of their autoantibodies that can still be quantified. Specific Aims include:1) To determine whether IgG-class autoantibodies for CNS markers are present in the blood sera of veterans with GWI compared with healthy GW veteran controls or compared with patients with irritable bowel syndrome (IBS). 2) To determine whether AChE inhibitor exposures during the war (i.e. low-dose sarin, pesticides, PB) are associated with IgG-class autoantibodies for CNS markers in veterans with GWI compared with veterans without GWI. 3) To determine whether IgG-class autoantibodies for CNS markers in veterans with GWI correlate with neuroimaging and cerebrospinal fluid markers in veterans with GWI compared with veterans without GWI. 4) To determine whether prior CNS insults (mTBI) are associated with Ig-G class autoantibodies for CNS markers in GW veterans with GWI compared with GW veteran controls.

- **Title: D-cycloserine –A Novel Treatment for Gulf War Illness (PI: Toomey)**
  Supporting agency: Department of Defense (GW140069)
  Performance period: 7/1/15-6/30/17
  Brief description of project's goals/ Specific aims: The overall objective of this GWI treatment study is to compare the efficacy of the randomized double-blind clinical treatment trial of DCS for the treatment of GWI. We hypothesize that the treatment trial of DCS will be effective in improving cognitive functioning in GW veterans with GWI, particularly memory functioning. Specific Aims: 1) To compare efficacy of the novel therapeutic approach of DCS in improving cognitive functioning in GW veterans with GWI. 2) To examine different time points in order to determine optimal timing of doses of DCS for positive effects on cognitive functioning. 3) To compare efficacy of DCS in improving mood, health symptoms and quality of life measures in GW veterans with GWI.

- **Title: Novel Interventions for Gulf War Veterans' Illnesses (PI: Niles and Mori)**
  Supporting Agency: DVA CSR&D
  Performance Period: 10/1/2015 – 9/30/2020
  Level of funding: $1,600,000
  Brief Description: This randomized trial will establish the effectiveness of a Tai Chi mind-body treatment in Veterans with GWI. Tai Chi is a traditional Chinese mind-body therapy that has been practiced for centuries. In the last decade, the PIs have demonstrated that Tai Chi can improve both physical health and psychological wellbeing in patients with a variety of chronic conditions. The long-term goal is to develop a safe, readily available, mind-body treatment to reduce pain and other chronic symptoms and enhance wellness in Veterans with GWI.

- **Title: Microtubule abnormalities underlying Gulf War Illness in neurons from human induced pluripotent cells (PI: Baas)**
  Supporting agency: Department of Defense (GW140086)
  Performance period: 7/1/15-6/30/17
  Level of funding: $1,026,352
  Brief description of project's goals/ Specific aims: The objective is to develop new immortal lines of pluripotent cells derived from peripheral blood mononuclear cells (PBMCs) of GW veterans themselves, so that an altered microtubule hypothesis (as well as other GWI hypotheses) can be tested. The other objective is to assess whether available microtubule-active drugs can correct these abnormalities and provide treatments for GWI. Specific aims are: 1. Develop human neurons or glial cells derived from human induced pluripotent stem cells (hiPSCs), originating from 15 GW veterans with GWI and 15 healthy GW veteran controls. 2. Develop a microtubule-based strategy to treat impaired nervous system functions in GWI.

Newly Submitted Studies

- CoQ10 Phase III trial - 4 site study submitted to DVA with Miami VA, GWIC, and other investigators (Klimas, PI).
- Ft Devens cohort cognitive, blood and neuroimaging assessment of brain antioxidant glutathione levels submitted with Boston VA, GWIC investigators (PI: Krengel and Sullivan).
- PON1 study with GWIC investigators and San Francisco VA investigators (PI: Chao)
- Epigenetic DNA methylation study submitted with GWIC and Naval Research Lab investigators (PI: Lin)
- Establish cohort of GW women veteran study with UMASS Lowell investigators (PI: Coughlin)
- Lipidomics and proteomics study with Roskamp investigators (PI: Abdullah)
- University of Colorado +naltrexone pain treatment New Investigator proposal (PI: Grace)

The consortium website (http://sites.bu.edu/gwic) is continually updated to disseminate news about new papers and studies related to Gulf War Illness.

OTHER ACHIEVEMENTS – None

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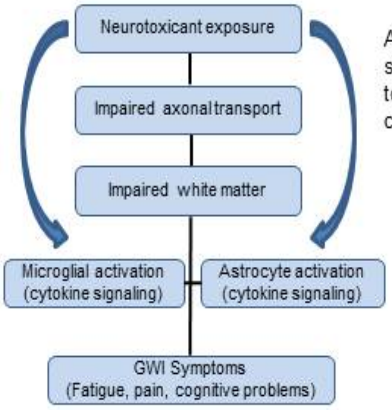
APPENDICES

Appendix A – Quad Chart

Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

Award Number: GW120037 / W81XWH-13-2-0072

PI: Dr. Kimberly Sullivan **Org:** Boston University Medical Campus **Award Amount:** $4,888,851

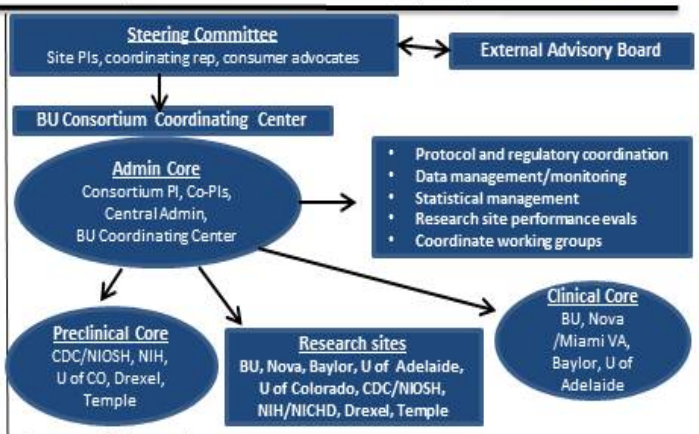


Hypothesized GWI Mechanisms

Approach

A series of clinical and preclinical studies to test whether GWI is related to chronic brain-immune activation and chronic inflammation.

- Clinical case-control studies will be conducted in parallel at 3 sites — Boston, Miami, and Central Texas and will include a total of 300 Gulf War veterans.
- Markers in blood, cerebrospinal fluid, brain imaging (advanced MRI, PET scans) and memory testing will be examined.
- Parallel preclinical studies will evaluate persistent effects of GW neurotoxicants in *in vitro* and rodent models of GWI.



Accomplishments: **1**-IACUC and ACURO approvals are in place for all sites **2**-IRB and HRPO approval obtained from BU, Miami VA and NOVA. **3**- Data and tracking systems, website finalized **4**-Laboratory methods have been established for immunologic assays. **5**-Preclinical studies have begun at all sites and have initial results. **6**- Subject recruitment has begun and first 17 subjects have been scheduled **7**- Six grant applications were recommended for funding for further collaborative research as a result of initial consortium planning meetings.

Timeline

Sept 2013 Start

Year

	1			2			3				4			Total
Months	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48		
Task1														
Task 2														
Task 3														
Task 4														
Task 5			50	30	30	25	33	33	34	55	0		n=300 human subjects	
Task 6						15	20	20	20	15	0		n=90 human subjects	
Task 7														
Task 8														

Goals/Milestones

FY13 Goal – Obtain necessary authorization prior to human/animal studies and preparation for consortium clinical/preclinical studies

- ☑ Kick-off meeting with GWIRP staff and study PIs
- ☑ Protocol preparation and initiation of approvals for animal/human use **(Task 1)**
- ☑ Creation of databases/manuals and data use agreements **(Task 2)**
- ☑ Prepare rodent dosing models and in vitro cell models **(Task 3)**

FY14 Goal –

- ☑ Perform preclinical cell/animal studies **(Task 4)**
- ☑Screening, recruitment, assessment of GW veterans at 3 sites **(Task 5)**

FY15 Goal – Recruitment and assessment for Boston CSF/PET studies **(Task 6-7)**

FY16 Goal – Statistical and validation analysis **(Task 7-8)**

Next External Advisory Board meeting scheduled for October 2015